http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (12): 2045-2050, 2004
ISSN 1028-8880
© 2004 Asian Network for Scientific Information

Mitogenic Effect of Bovine β-lactoglobulin and its Proteolytic Digests on Mouse Spleen Resting Cells

R. Mahmud, M.A. Matin and [1]H. Otani
Department of Dairy and Poultry Science, Chittagong Government Veterinary College,
Pahartali, Chittagong-4202, Bangladesh
[1]Laboratory of Food Bioscience, Graduate School of Agriculture, Shinshu University,
8304 Minamiminowa-mura, Nagano-ken 399-4598, Japan

Abstract: Bovine milk β-lactoglobulin was found to induce proliferation of mouse resting spleen cells. Hence, the mitogenic activity was investigated in detail. The cell growth activity was measured by a colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT). A reduced S-carboxymethylated β-lactoglobulin retained mitogenic activity towards mouse spleen cells and the mitogenic activity increased noticeably after digestion with gastrointestinal proteinases, in particular pancreatin. The pancreatin digest also induced proliferation of U266 cells, a human B cell line while it little influenced the growth of a human B cell line, Ball and human T cell lines Jurkat or Molt-3. These results suggest that bovine β-lactoglobulin may involve a mitogenic sequence(s) against some lymphocytes by the action of intestinal proteinases.

Key words: β-lactoglobulin, proteolytic digests, mitogenic activity, mouse spleen cells

INTRODUCTION

Milk proteins are known to serve different physiological functions in addition to provide essential nutrients for the young mammals. Milk contains a variety of defense factors including antibody, lactoferrin, lysozome and many soluble antimicrobial factors, which provide passive immunity to the newborn[1]. It is also known that milk components contribute to the development of active immune system by regulating the cells of the immune system[2-8]. Otani and co-workers systematically studied the immunomodulatory properties of milk proteins and demonstrated that different casein components have a variety of suppressive and stimulatory effects on mononuclear cells[9-11]. In general, the immunomodulatory functions of these components become more evident as they are digested with intestinal proteolytic enzymes[12].

Kappa-casein glycomacropeptide (residues 106-169 of κ-casein, CGP), released from its parent casein by the action of stomach proteinases such as chymosin and pepsin, have been found to inhibit the mitogen-induced proliferation of mouse spleen lymphocytes and rabbit Peyer's patch cells[9]. The suppressive mechanism is attributable to two ways: by the induction of IL-1 receptor antagonists (IL-1ra) secretion via binding of CGP to monocyte/macrophage and by suppressing IL-2 receptor expression on CD4+ T lymphocytes via binding of CGP to the cell surfaces[10,13]. Recently, Matin *et al.*[14] identified an apoptosis-inducing peptide for lymphocytes, κ-casecidin, in a trypsin digest of bovine κ-casein. These immunosuppressing peptides may contribute to the development of host defense of breast-fed infants by playing anti-inflammatory role in the intestine.

Some peptides released from calcium-sensitive caseins by the action of proteolytic enzymes are known to stimulate cells relating to immune system. Several small peptides derived from β-casein have been found to enhance macrophage phagocytic activity[15]. Recently, Hata *et al.*[16] reported that bovine casein phosphopeptides, such as bovine αs1-casein (59-79) and bovine β-casein (1-25), showed mitogenic activity towards mouse spleen and rabbit Peyer's patch cells cultures. A phosphopeptide having at least two-phosphoserine residue i.e. SerP-X-SerP has been identified as the immunostimulatory active center of the CPP. From these observations Otani *et al.*[8] has proposed that casein phosphopeptides liberated from calcium-sensitive caseins by the action of proteolytic enzymes may enhance mucosal immune response in man and livestock.

Corresponding Author: Dr. M.A. Matin, Assistant Professor, Department of Dairy and Poultry Science,
Chittagong Government Veterinary College, Pahartali, Chittagong-4202, Bangladesh
E-mail: matinma2000@yahoo.com

In contrast to caseins, a few immunoregulatory peptides have been characterized from whey proteins. Miyauchi *et al.*[12] reported that pepsin hydrolysis of bovine lactoferrin produced peptides that enhanced proliferation of murine spleen cells. Lactoferricin, which was identified as bactericidal peptide in the pepsin digest of lactoferrin, is demonstrated to enhance phagocytic activity of human neutrophil and to induce apoptosis towards human monocytic tumor cells[17,18].

On the other hand, β-lactoglobulin (β-LG) comprises about 50% of the bovine whey proteins. It forms dimer at physiological pH, each monomer being an 18-kDa globular protein of 162 amino acids containing five-cysteine residue[19]. The molecule contains two disulfide and one free sulfhydryl groups and is considered one of the major allergenic components in human milk, because it is not an indigenous part of human milk. Recently, Wong *et al.*[20] found that bovine whey proteins displayed immunostimulatory activity towards mouse spleen cells, and suggested that the immunostimulatory activity of the whey protein was due to β-LG. More recently, the authors found that bovine β-LG possessed mitogenic activity towards mouse spleen cells. Thus, in the present work, the authors characterized the mitogenic activity of bovine β-LG in cell cultures.

MATERIALS AND METHODS

This experiment was done in accordance with the guidelines for Regulation of Animal Experimentation in the Faculty of Agriculture of Shinshu University and according to the Law No. 105 and Notification No. 6 of the Japanese Government.

Reagents: Pepsin (porcine stomach mucosa), trypsin (bovine pancreas), chymotrypsin (bovine pancreas), pancreatin (bovine pancreas) and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal calf serum (FCS) and RPMI-1640 were obtained from Gibco BRL (Grand Island, USA). Penicillin G and streptomycin sulfate were purchased from Meiji Seika (Tokyo). The other chemicals used were of the highest analytical grade commercially available.

Preparation of β-LG and its enzymatic digests: β-LG was prepared from fresh whole milk obtained from Holstein cows according to the method of Aschaffenburg and Drewry[21].

The β-LG was reduced S-caboxymethylated according to the procedure described in Otani and Tokita[22]. Reduction of β-LG was carried out in 8 M urea by exposure to 2-mercaptoethanol followed by carboxymethylation with access iodoacetic acid. The resultant derivatives were dialyzed against water, lyophilized and then treated twice more in 8 M urea with 2-mercaptoethanol and iodoacetic acid in the same manner as described above.

Digestion of β-LG with pepsin was carried out in 0.01 M hydrogen chloride at 45°C (pH 2.0, E/S = 1/100, w/w), while that with trypsin, chymotrypsin or pancreatin was performed in 0.01 M sodium phosphate buffer containing 0.15 M sodium chloride (pH 8.0, PBS, E/S = 1/100). After the reaction at 45°C for 3 h, the pH of each reaction mixture was adjusted to 7.2 with 0.3 M sodium hydroxide and then the reaction mixture was subjected to ultrafiltration at 4°C on an Amicon Ultrafiltration Cell Model 52 (Amicon Corporation, Danvers, USA) with diafilter A-15T having a 15 kDa exclusion (Advantec, MFS Inc., Tokyo) to remove proteinases and intact β-LG. The filtrate was recovered as pepsin, trypsin, chymotrypsin or pancreatin digest. The amount of peptide in the digest was determined by the method of Lowry *et al.*[23] and the degree of digestion was expressed as:

Degree of digestion (%) = 100×(amount of peptide in the prepared digest/amount of β-LG used as substrate).

Cell cultures

Mouse spleen cells: Male C_3H/HeN mice, 6 weeks of age, were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Mice were sacrificed by cervical dislocation and spleens were removed in sterile condition. Single cell suspension was prepared by gentle teasing of the tissues in cold RPMI-1640 containing 100 units mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin. After being washed three times in the medium the cells were resuspended in the medium at a concentration of 6.0×10^6 cells mL^{-1} and were used as mouse spleen cell suspension. Cell cultures were set up in quadruplicate on flat-bottomed microtiter plates (Falcon Labware, Oxnard, USA). Into each well were placed 100 μL of cell suspension, 20 μL of a test protein or peptide solution dissolved in PBS. Final concentrations were: spleen cells, 5×10^6 viable cells mL^{-1}; test protein or peptide 0 to 500 μg mL^{-1}; penicillin 100 units mL^{-1}; streptomycin 100 μg mL^{-1}. The mixtures were cultured at 37°C in a humidified 5% CO_2-95% air atmosphere for ~72 h.

Human cell lines: Jurkat Clone E6-1 cells or MOLT-4 (human T cell lines) and BALL cells or U266 cells (human B cell lines) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were grown in RPMI-1640 supplemented with 10% heat

inactivated FCS, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C in a humidified 5% CO_2- 95% air atmosphere. Cells grown were collected and washed with PBS (pH 7.2). Washed cells were resuspended in cold RPMI-1640 containing 100 units mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin. Cell cultures were set up in quadruplicate on flat-bottomed microtiter plates (Falcon Labware, Oxnard, USA). Into each well were placed 100 μL of cell suspension, 20 μL of a test protein or peptide solution dissolved in PBS. Final concentrations were: human cell lines, 1.2×10^5 viable cells mL^{-1}; test protein or peptide 0 to 500 μg mL^{-1}; penicillin 100 units mL^{-1}; streptomycin 100 μg mL^{-1}. The mixtures were cultured at 37°C in a humidified 5% CO_2-95% air atmosphere for ~72h.

Determination of cell proliferation: At the end of the cultivation period, the proliferative response of cultured cells was estimated by colorimetric MTT (tetrazolium) assay (Mosmann, 1983). The principle of the method was based on the reduction of MTT by metabolically active cells to insoluble coloured formazon. The absorbances of formazan formed were measured spectrophotometrically at 570 nm by a Bio-Rad model 450-microplate-reader (Bio-Rad Laboratories, Hercules, CA94547, USA). The results were expressed as mean absorbance at 570 nm (MTT value) or mean cell growth activity±standard deviation. Cell growth activity was calculated as follows:

$$\text{Cell growth activity (\%)} = 100 \times \left(\frac{\text{MTT value in the culture with test peptide}}{\text{MTT value in the culture without test peptide}} - 1\right)$$

Statistical analysis: The results of cell proliferation was expressed as mean absorbance at 570 nm (MTT value)±standard deviation or mean cell growth activity±standard deviation. The significance of difference was tested by Student's t-test. The result presented were the representative of three separate repeated experiments.

RESULTS AND DISCUSSION

Effect of β-LG on mouse spleen resting cells: C_3H/HeN mouse spleen cells were cultured with β-LG for different incubation period and cell conditions were determined by colorimetric MTT assay. As shown in Fig. 1, β-LG increased MTT values at a concentration of 500 μg mL^{-1}. Maximum increase occurred after 72 h of cultivation. These results indicate that β-LG possessed a mitogenic activity towards mouse spleen resting cells. Similarly, the spleen cells were cultured with reduced S-carboxymethylated β-LG and cell conditions were determined. As shown in Fig. 2, reduced S-carboxymethylayed β-LG induced cell proliferations as well as the native protein. These results suggest that the mitogenic activity of β-LG depends on primary structure and not conformational.

Effect of β-LG digests on mouse spleen resting cells: Mouse spleen cells were cultured with pepsin, trypsin, chymotrypsin and pancreatin digests of β-LG and cell conditions were determined. As shown in Fig. 3, all the digests induced proliferation of mouse resting spleen cells. In particular, the activity of the pancreatin digest was strongest, since 100 μg mL^{-1} of the concentration displayed a high mitogenic activity. These results indicate that the mitogenic activity of β-LG depends on some amino acid requences of its molecule.

Three human cell lines were cultured with the pancreatin digest and cell conditions were determined. As shown in Fig. 4, the digest induced significant cell growth of a human B cell line, U266 cells at a concentration of 500 μg mL^{-1} but hardly influenced the growth of a human B cell line, Ball and human T cell lines, MOLT-4 and Jurkat cells. These results suggest that bovine B-LG possess a mitogenic activity towards some B cells.

Food consumption is associated with immune system in several ways. Good nutritional status is necessary for proper functioning of the immune system, since food provides nutrients essential for producing the elements of the immune system. It is known that milk is rich in essential nutrients, growth factors and immunomodulatory components, which influence growth, development and immune status of newborn infants. In addition, milk proteins are sources of biologically active peptides, which could be liberated from the parent proteins by gastrointestinal digestion or food processing. A group of such peptides have been identified in the enzymatic digests of milk proteins and implicated on the stimulation of immune system. Otani *et al.*[9,13] reported that calcium-sensitive and insensitive caseins produced stimulatory and suppressive activities towards proliferation and/or immunoglobulin production of mouse spleen cells when the caseins were digested with gastrointestinal proteinases. Moreover, it is reported that some peptides liberated from caseins by tryptic digestion exhibited cytotoxic activity towards eukaryotic and/or prokaryotic cells[18,25].

The results of the present study demonstrated that β-LG possessed a mitogenic activity towards some B cells and the activity is attributable to some sequences of β-LG molecule. Ingestion of milk results in exposure of the milk proteins to proteolytic enzymes in gastrointestinal tract.

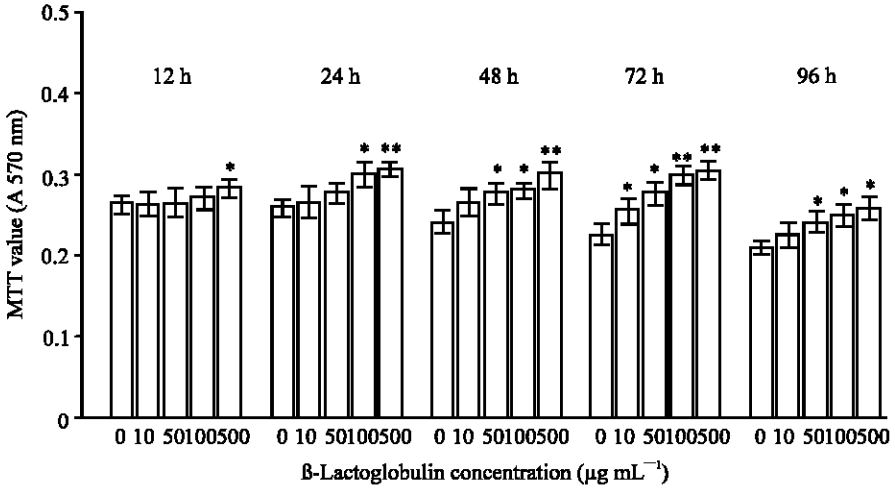

Fig. 1: Effect of native β-LG on mouse resting spleen cells. Values are mean with their standard deviations represented by vertical bars from n = 4. MTT values between control group (β-LG, 0) and test group (β-LG, 10~500) were significantly different: * $p<0.05$, ** $p<0.01$, *** $p<0.001$

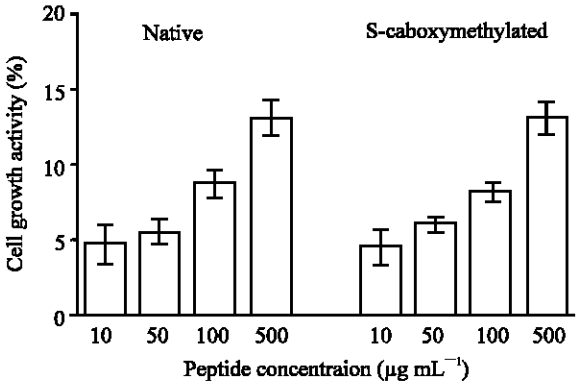

Fig. 2: Effect of S-carboxymethylated β-LG on mouse resting spleen cells

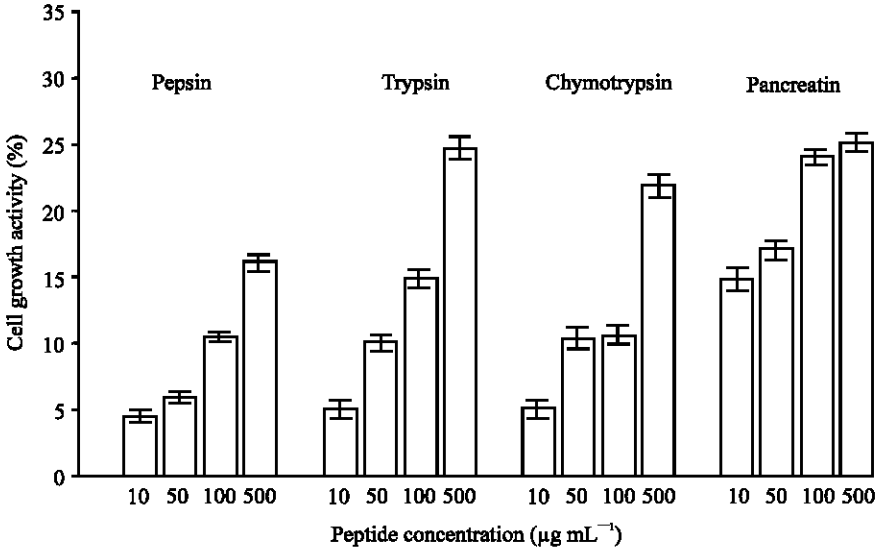

Fig. 3: Effects of proteinase digests of bovine β-LG on mouse spleen cells. Values are mean with their standard deviation from n = 4

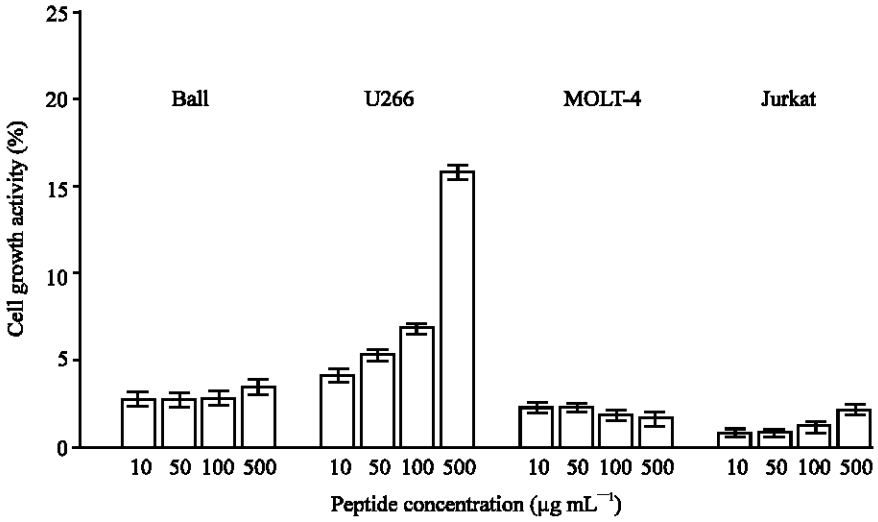

Fig. 4: Effect of pancreatin digest of bovine β-LG on human cell lines

Pepsin, trypsin and chymotrypsin are major proteinases in gastrointestinal tracts, and pancreatin involves various enzymes such as trypsin and chymotrypsin. The results obtained in the present work shows that all of the pepsin, trypsin, chymotrypsin and pancreatin digests display mitogenic activity stronger than native β-LG. Therefore, it is suggested that the mitogenic peptides may have valuable role in the development of immune system in calves. On the other hand, this study could not identify any specific peptides responsible for the mitogenic activity. Further study is in progress to characterize mitogenic activity of the β-LG.

REFERENCES

1. Goldman, A.S., 2000. Modulation of gastrointestinal tract of infants by human milk. Interface and interactions. An evolutionary perspective. J. Nutr., 130: 4265-4315.
2. Lönnerdal, B., 1985. Biochemistry and physiological function of human milk proteins. Am. J. Clin. Nutr., 42: 1299-1317.
3. Julius, M.H., M. Janusz and J. Lisowski, 1988. A colostral protein that induce growth and differentiation of resting B-lymphocytes. J. Immunol., 140: 1366-1371.
4. Otani, H., M. Monnai and A. Hosono, 1992. Bovine κ-casein as inhibitor of the proliferation of mouse splenocyte induced by lipopolysaccharide stimulation. Milchwissenschaft, 47: 512-515.
5. Pabst, H.F., 1997. Immunomodulation by breast-feeding. Pediat. Infect. Dis. J., 16: 991-995.
6. Gill, H.S., F. Doull, K.J. Rutherfurd and M.L. Cross, 2000. Immunoregulatory peptide in bovine milk. British J. Nutr., 84: S111-S117.
7. Otani, H., Y. Kihara and M. Park, 2000. The immunoenhancing property of dietary casein phosphopeptide preparation in mice. Food Agric. Immunol., 12: 165-173.
8. Otani, H., T. Watanabe and Y. Toshiro, 2001. Effect of bovine β-casein fragment 1-28 and its chemically synthesized partial fragments on proliferative response and immunoglobulin production in mouse spleen cell cultures. Biosci. Biotech. Biochem., 65: 2489-2495.
9. Otani, H. and M. Monnai, 1993. Inhibition of proliferation of mouse spleen lymphocytes by bovine milk κ-casein digests. Food Agric. Immunol., 5: 219-229.
10. Otani, H., Y. Horimoto and M. Monnai, 1996. Suppression of interleukin-2 receptor expression on mouse CD^{4+} T cells by bovine κ-caseinoglycopeptide. Biosci. Biotech. Biochem., 60: 1017-1019.
11. Otani, H., K. Nakano and T. Kawahara, 2002. Stimulatory effect of dietary casein phosphopeptides preparation on mucosal IgA response of mice to orally ingested lipopolysaccharide from *Salmonella typhimurium*. Biosc. Biotech. Biochem., 67: 2489-2495.
12. Miyauchi, H., I. Shinoda Y. Fukuwatari and H. Hayasawa, 1998. Immunostimulatory effect of bovine lactoferrin pepsin hydrolysate on murine splenocyte and Peyer's patch cells. J. Dairy Sci., 80: 2330-2339.

13. Otani, H. and M. Monnai, 1995. Induction of interleukin-1 receptor antagonist-like component produced from mouse spleen cells by bovine κ-caseinoglycopeptide. Biosci. Biotech. Biochem., 59: 1166-1168.
14. Matin, M.A., M. Monnai and H. Otani, 2000. Isolation and characterization of a cytotoxic pentapeptide, κ-casecidin, from bovine κ-casein digested with bovine trypsin. Ani. Sci. J., 71: 197-2007.
15. Kayser, H. and H. Meisel, 1996. Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk protein. FEBS Lett., 383: 18-20.
16. Hata, I., S. Higashyama and H. Otani, 1998. Identification of a phosphopeptides in bovine casein digest as a factor influencing proliferation and immunoglobulin production in lymphocyte cultures. J. Dairy Res., 65: 569-578.
17. Miyauchi, H., M. Hashimoto, I. Nakajima, I. Shinoda Y. Fukuwatari and H. Hayasawa, 1998. Bovine lactoferrin stimulate the phagocytic activity of neutrophils: identification of its active domain. Cell. Immunol., 187: 34-37.
18. Yoo, Y., R. Watanabe, Y. Koike, M., Mitobe, K. Shimazaki, S. Watanabe and I. Azuma, 1997. Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: Involvement of reactive oxygen species. Biochem. Biophys. Res. Commun., 237: 624-628.
19. Susan, G.H., S.M. Alan and S. Lindsay, 1989. β-lactoglobulin. In: Fox, P.F. (Ed.) Development in Dairy Chemistry-4. Elsevier Science Publishers, Ltd. London, pp: 141-53.
20. Wong, K.F., N. Midleton, M. Montgomery, M. Dey and R.I. Carr, 1998. Immunostimulation of murine spleen cells by materials associated with bovine milk fractions. J. Dairy Sci., 81: 1825-1832.
21. Aschaffenburg, R. and J. Drewry, 1957. Improved method for the preparation of crystalline β-lactoglobulin and α-lactalbumin from cows' milk. Biochem. J., 65: 273-277.
22. Otani, H. and F. Tokita, 1980. Effects of treatments with urea and some chemical reagents on the antigenicity of β-lactoglobulin. Japan. J. Zootech. Sci., 51: 17-20.
23. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randal, 1983. Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265-275.
24. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.
25. Matin, M.A. and H. Otani, 2002. Cytotoxic and antimicrobial properties of chemically synthesized κ-casecidin and its partial peptides. J. Dairy Res., 69: 329-334.
26. Migliore-Samour, D. and P. Jollés, 1988. Casein, a prohormone with an immunomodulting role for the newborn? Experimentia, 44: 188-193.