In vivo antibacterial activity of whey protein derived from fermented milk of Iraqi buffalo

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Abstract

The present study aims to prepare fermented buffalo's milk rich with low molecular weight peptides by using lactic acid starters as a mixture. Skim milk sample was inoculated with 5% of the starter. The growing number of starter and anti-bacterial activity were studied after 24 hours of incubation. Protein and peptide concentration were determined before and after fermentation, then biological active peptides were isolated or separated and purified by gel filtration column of Sephadex G25. Finally antibacterial activity of the isolated peptides was study in vivo. The results of chemical analysis of fresh and fermented milk showed that the concentrations of protein were 0.817mg/ml and 0.501mg/ml before and after fermentation, respectively either peptide concentration were 0.4mg/ml before fermentation and 0.805mg/ml after fermentation. The number of starter was determining during the fermentation process after 6, 12, 18 and 24 hours of incubation and found an increase in the number of lactic acid bacteria. The initiation number was 6.2×10^5 but after the 24 hours the number increased of up to 1.3×10^6. Number of lactic bacteria decreased after 24 hours with the increase in the concentration of lactic acid combined with low pH value. Colonies of lactobacilli were isolated from fermented buffalo milk and was characterized by the typical characteristics for the purpose of a rating based on morphological and cultural characters. Gel filtration gave seventy-eight fractions. And depending on the absorbency on wavelength 280 were obtained four peaks, each peak represents a fraction. Peptide concentration was determined in each fraction, these concentrations were (0 and 0243 and 0902 and 0632) mg / ml of fraction 1, 2, 3 and 4, respectively. Fraction three contained a high concentration of peptide. The antibacterial activity of the third fraction was estimated. The results showed that the bioactive peptides of fermented milk have good efficacy in the treatment of diarrhea in laboratory animals.
Key words: Antibacterial peptide, whey, protein, milk, buffalo.

الدراسة في الجسم الحي للفعالية المضادة للجراثيم لبروتين المصل المشتق من الحليب المتخمرة للجاموس العراقي

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تهدف الدراسة الحالية الى تحضير حليب الجاموس المتخمرة الغني بالببتيدات منخفضة الوزن الجزيئي باستخدام زرع خليط من بادئ حامض اللبنيك. لقحت عينة الحليب الخالي من الدسم ب 5٪ من البادئة. درست تزايد اعداد البادئة والنشاط المضاد البكتيري بعد 24 ساعة من الحضانة. حدد تركيز البروتين و الببتيدات قبل وبعد التخمير، ثم فصلت ونقيت الببتيدات النشطة بيولوجيا بعمود ترشيح الهلام باستخدام Sephadex G25. وأخيرا تم دراسة النشاط المضاد للبكتيريا العائد للببتيدات المعزولة في الجسم الحي. أظهرت نتائج التحليل الكيمائي للحليب الطازج والمتخمر بان معدل تركيز كل من البروتين 0.817 و0.501 قبل وبعد التخمير على التوالي اما تركيز البيبتيدات هو 0.4 قبل التخمر و 0.805 بعد التخمر. تم تحديد عدد البكتيريا البادئة الحية خلال عملية التخمير بعد 6 و 12 و 18 و24 ساعة من الحضن ووجد زيادة في عدد بكتريا حامض اللاكتيك. اذ كان العدد الاولي 6.2×10^5 بكتريا وبعد 24 ساعة ازداد العدد ليصل الى 1.3×10^6. وقد انخفض عدد البكتريا اللبنية بعد 24ساعة مع زيادة تركيز حامض اللاكتيك مترافقا مع انخفاض قيمة الاس الهيدروجيني. تم عزل مستعمرات العصيات اللبنية من حليب الجاموس المتخمر وكانت تتصف بصفات نموذجية لغرض التصنيف المعتمد على الصفات الزرعية والشكلية. اعطى الترشيح بالهلام 78 جزء. واعتمادا على امتصاصية الطول الموجي 280 تم

الحصول على اربعة قمم كل قمة تمثل جزء. تم تحديد تركيز البيبتايد في كل من هذه الاربعة الاجزاء حيث كانت هذه التراكيز (0 و 0,243 و 0,902 و 0,632) ملغم / مل للجزء 1و2و3و4 على التوالي. ظهر الجزء الثالث انه يحتوي على تركيزعالي من البيبتيدات. تم تقدير النشاط المضاد للبكتيريا للجزء الثالث. أظهرت النتائج بان الببتيدات النشطة بيولوجيا للحليب المتخمرة لها فعالية جيدة في علاج حالات الإسهال في الحيوانات المختبرية.

الكلمات المفتاحية: الببتيد المضاد للجراثيم ، مصل اللبن ، البروتين ، الحليب ، الجاموس.

Introduction

Milk is an excellent source of highly valuable proteins which are in general divided into caseins and whey proteins. Caseins and whey proteins comprise approximately 80% and 20%, respectively of total milk proteins (1). Numerous health advantages of milk protein derived bioactive peptides have been claimed for commercial interests in the environment of health sustaining-functional foods (2). Moller *et al*., (2008) (3) defined bioactive peptides as substances that can affect the biological processes of the body functions with beneficial effects. Milk proteins have been recognised as potential sources of biological active peptides that are latent and encrypted in their native form. These biologically active peptides can be generated and activated by different mechanisms including: (a) protein hydrolysis by digestive enzymes (b) food processing and (c) proteolytic activity by enzymes derived from microorganisms, especially lactic acid bacteria. Potent biologically active peptides have been isolated from a number of fermented dairy products such as cheese, fermented milk and yoghurt (2). Due to growth requirements, dairy starter cultures have developed highly sophisticated proteolytic system capable of breaking down milk proteins, mainly αs1- and β-caseins. The lactic acid bacteria (LAB) proteolytic structure and their activities in dairy products including yoghurt and cheese have been studied extensively (4).

Materials and methods

Buffalo milk samples collection:

Buffalo's milk sample was obtained from Iraqi buffalo. Sample was immediately kept at 4 ^{o}C during transporting to the laboratory. Each fermentation batch (500 ml) was equilibrated for 1 h. at the fermentation temperature (40^{o}C) in a water path before inoculation with the starter bacterial cultures for this purpose.

Preparation of fermented milk:

Skim milk samples were inoculated with the starter culture containing mixed cultures of the local species of starter. These fermented samples were incubated at 37^{o}C for 42 h. The viable cells of lactic acid bacteria in the fermented milk were counted using modified Rogosa sharpe agar (MRS agar) (Difco). The whey fraction was obtained by adjusted the pH of fermented milk to 4.6 with (1 N HCl) or (1 N) NaOH. Then it was centrifuged at 10,000 xg for 20 min. The supernatant was adjusted to pH 8.3 with (1N NaOH), then centrifuged again at 10,000 xg for 10 min. The final supernatant was used as the whey fraction (5).

Extraction of whey protein:

The whey protein extract was prepared after fermentation of skimmed milk. Fermented milk was acidified to a pH of 4.2 by adding of 2N HCl. The solution was centrifuged at a speed of 10000 *g* at 4°C for 30 minutes. Casein (the sediment) was removed from whey by filtration using No.1 Whatmann filter paper. Whey acid was neutralized to pH 6.8 by addition 2 N NaOH, then centrifuged at 10000*g* at 4°C for 30 minutes. The whey supernatant obtained was neutral (6).

Estimation of protein concentration:

The protein concentration of the fresh and fermented milk was determined by the method of Bradford. Bovine Serum Albumin (BSA) was used as standard protein to prepare the standard curve and Coomassie Brilliant Blue G-250 as the reagent (7).

Determination of peptide concentration methods:

The peptide concentration of the fresh and fermented milk was determined by the method of o-phthaldialdehyde (OPA). Glutathion was used as standard matter to prepare the standard curve and OPA as the reagent (7).

Separation of low molecular weight bioactive peptide from fermented milk by gel filtration:

Peptides were separated by passing of whey protein of fermented milk through the Sephadex G25 column in dimension 40 X 1.6 cm. The recovered parts of the column were collected in a speed flow 30 ml / hour, the optical absorption of the washing and recovery parts were measured on wavelength 280 nm to determinate the fraction numbers according to the curves then the fractions were concentrated by a rotary evaporator at a temperature of 50 ^{o}C (8).

Determination of antibacterial activity of bioactive peptide of the third fraction *in vivo*:

To study the inhibitory effect of bioactive peptide towards *E. coli*, *Klebseila pneumonia* and *Staph. aureus* that were causing diarrhea in rats (*In vivo*) following the method of (9). Animals were divided into four groups; each group contained five rats as following:

Group A: Five animals were administrated (1ml) distillated water by gastric tube, for (4, 8, and 12) days.

Group B: Five animals were administrated (1ml) peptides by gastric tube for (4, 8, and 12) days.

Group C: Fifteen animals were administrated 10^5cfu pathogenic bacteria in (1ml) by gastric tube for first four days (Five animals for each strain).

Group D: Animals were administrated 10^5cfu pathogenic bacteria in (1ml) by gastric tube for first four days (five animals for each strain), in fifth day; the animals were administrated with the first peptide dose (1 ml) and repeated every day until the 12th day.

Results

Peptide and protein content of fresh and fermented buffalo's milk:

Results were showed the concentrations of protein and peptide in both fresh and after milk fermentation that estimated by OPA and Bradford methods. Peptide concentration was increased during the fermentation time progresses but protein concentration was decreased due to bacterial enzymatic activity (Table 1).

Table (1): The proteins and peptides concentration per ml of a fresh buffalo's milk.

Materials	Mg/ml (mean)	
	Before fermentation	After fermentation
Protein	0.817	0.501
Peptide	0.4	0.805

The viable counts of starter bacteria cultures during fermentation with Isolation and identification of *Lactobacillus* spp. from fermented buffalo's milk:

Lactic acid starters (LAS) were isolated from fermented milk on MRS agar initially. All isolates were obtained and identificated morphologically by the colony characteristics of the isolates obtained, along with their Gram reaction and microscopic examination. Changes in the viable counts of the starter cultures of lactic acid bacteria throughout fermentation are presented in table (2).

Table (2): The total number of lactic acid starter after fermentation (6, 12, 18 and 24hrs.).

Time / Count	6hrs	12hrs	18hrs	24hrs
No. of bacteria	6.2×10^{5}	7.4×10^{5}	1.1×10^{6}	1.3×10^{6}

The bacterial number was increased with time progresses until 24hrs, after that the number of starter bacteria was decreased due to metabolites concentration and reduction of nutrition. The common bacteria were *Lactobacillus* spp. and colonies that isolated from fermented buffalo's milk with typical characteristics (white, small with entire margin) were picked up and transferred to nutrient broth which was then subjected to classification based on morphological and biochemical characters. All strains were reacting positively to Gram stain. *Lactobacilli* spp. were long rods but sometimes were coco-bacilli. *Lactobacillus* showed negative result to motility test because they did not possess flagella, while

citrate and indole and catalase were negative. All isolates were isolated from fermented milk were found to ferment glucose and lactose to lactic acid.

Determination of fractions as the peak according to the presence of low molecular weight peptide:

These peaks that were determined according to absorbency of each fraction were measured on wavelength 280nm. Three peaks were obtained. The first peak did not contain any peptide so that it did not have the inhibitory activity against any type of pathogenic bacteria. It was represent the first fraction. The second peak contained peptide concentration (0.243) mg / ml and had given the effectiveness of inhibitory action against each type of pathogenic bacteria. The third peak contained a higher peptide concentration (0.902) mg / ml. which showed the higher antibacterial activity against bacteria. The 4th peak contained a peptide concentration (0.632) mg / ml and had given the effectiveness of inhibitory action. The peptide concentrations of each fraction were (0, 0.243, 0.902 and 0.632) mg/ml of fraction 1, 2, 3 and 4 respectively. The third fraction showed the highest concentration then the forth fraction (Fig. 1).

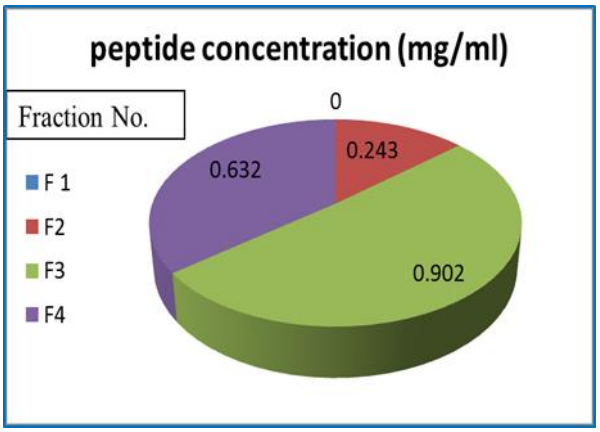

Fig. (1): The peptide concentrations of each fraction.

***In vivo* study of Antibacterial activity of the third fraction:**

The results showed the antibacterial activity of the third fraction *in vivo* because it had the higher peptide concentration and antibacterial zone. The infected rats with *E. coli* were more effective than that infected with *K. pneumonia* and *Staph. aureus* (Table 3).

Table (3): Antibacterial activity of the third fraction *in vivo*.

Treatment	Group A	Group B	Group C			Group D		
			E. coli	*K. pneumonia*	*Staph aureus*	*E. coli*	*K. pneumonia*	*Staph aureus*
1 day	All live	All live	All live	All live	All live	All live	All live	All live
4 days	All live	All live	All sickness	All sickness	All sickness	All sickness	All sickness	All sickness
8 days	All live	All live	2 dead	1 dead	3 dead	All sickness	All sickness	All sickness
12 days	All live	All live	All dead	All dead	All dead	All live	All live	All live

Discussion

The study showed the means of peptide and protein per ml of fresh and fermented buffalo's milk and referred to increase of peptide concentration due to proteolysis by lactic acid bacteria enzymes and also referred to buffalo's milk which had higher than cow's milk in these concentration because they depend on many factors such as analysis methods, geographical position, feeding, as well as management, age and lactation stage, this was agreement with results of (10). Lactic acid starters (LAS) were isolated from fermented milk on MRS agar initially. All isolates were obtained and identificated morphologically and biochemically. The common bacteria were *Lactobacillus* spp. and colonies were isolated from fermented buffalo's milk with typical characteristics and biochemical reactions. This result was resembled to that obtained by (11). Lactic acid starter count was decreased after 24hrs. due to the increase of the lactic acid

concentration that companied with decreases the pH value. Depression in the pH value in the buffalo's milk was higher than the depression in cow's milk due to the decrease in the buffering capacity and variation in the milk composition (12). The results showed the antibacterial activity of the third fraction *in vivo* and such result was similar to the study that carried out in Morocco by Kalalou who studied the activity of LAB diet products on some gram positive and negative pathogenic bacteria as *E.coli*, *Pseudomonas aeroginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus cereus* and the inhibition zones were in the range of 1.4 to 2.8 cm. Probiotic microorganisms interacted with pathogenic bacteria and bowel micro-flora by the production of antimicrobial substances and competitive inhibition (13).

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