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Effect of Bovine Lactoferrin and Casein Peptide Powder on Microbial Growth and Glucose Utilization by Microorganisms in Pork Meat During Storage at 4°C

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Abstract: In this research, the effect of Lactoferrin and Casein peptide on antimicrobial activities and glucose utilization by microorganisms in hot-boned (4-5 h after death) pork meat during storage at 4°C for five days was examined. Total plate count was lower for sample with added Lactoferrin and Casein peptide than control sample. Meat sample with added Lactoferrin had lower plate count than sample with added Casein peptide. High Pressure Liquid Chromatography (HPLC) was also used to detect pork meat glucose also known as freshness index of meat and the results showed that glucose content increased during storage from day 1 to day 3 then slightly decreased at day 5 with the addition of Lactoferrin and Casein peptide and the differences were not significant ($p<0.05$), however it was found to decrease during storage for control sample. The addition of Lactoferrin and Casein peptide decreased the bacterial counts at days 0, 1, 3 and 5 and increased glucose content during storage of hot-boned pork meat at 4°C.

Key words: Freshness, glucose content, meat, preservatives, shelf-life, milk protein

INTRODUCTION

Lactoferrin: Lactoferrin (LF) is an iron-binding glycoprotein that mainly exists in mammalian milk. Lactoferrin has the ability to bind ferrous iron ions (Baveye *et al*., 1999; Naidu, 2002) and its bacteriostatic effect is probably due to that ability to sequester free iron required for microbial growth (Oram and Reiter, 1968; Weinberg, 1975). Concentration of iron ions are important catalysts of lipid oxidation, which could be inhibited by lactoferrin (Naidu, 2000, 2002). In particular, lactoferrin provides antibacterial activity to human infants.

It is a multifunctional iron glycoprotein which is known to exert a broad-spectrum primary defense activity against bacteria, fungi, protozoa and viruses. Its iron sequestering property is at the basis of the bacteriostatic effect, which can be counteracted by bacterial pathogens by two mechanisms: the production of siderophores which bind ferric ion with high affinity and transport it into cells, or the expression of specific receptors capable of removing the iron directly from lactoferrin at the bacterial surface (Orsi, 2004).

Its antimicrobial activities include stasis, cidal, adhesion-blockade, cationic, synergistic and opsonic mechanisms against different bacteria (gram-positive and Gram-negative, rods and cocci and aerobes and anaerobes), DNA and RNA viruses, a variety of yeasts, fungi and parasites. In addition, LF expresses anti-inflammatory and immunomodulatory properties that enhance effectiveness of its antimicrobial action. LF has found its way into infant formula and health foods in SE Asia. It is used as a therapeutic and prophylactic agent for control of intestinal illnesses.

A number of efficacy studies and clinical trials are ongoing in various laboratories with over 100 patents filed on this molecule in the last ten years. LF is emerging as one of the leading natural microbial blocking agents in food safety and preservation.

In the U.S.A., lactoferrin is permitted at levels of 65.2 mg/kg in beef (Naidu, 2002). In Taiwan, there is no regulation for lactoferrin. Lactoferrin may be used in special nutritional foods "only for supplementing foods with an insufficient nutritional content and may be used in appropriate amounts according to actual requirements" (Chiu and Kuo, 2006).

Many scientists have indicated that lactoferrin can inhibit various microorganisms (Oram and Reiter, 1968; Weinberg, 1975; Naidu, 2000, 2002), but inhibition has not been clearly documented. Only limited information has been reported on the effect of lactoferrin on lipid oxidation (Huang *et al*., 1999) and antimicrobial properties in food systems (Naidu, 2002). The effects of this additive on pork meat glucose utilization by microorganisms are not known.

Casein peptides: Milk proteins constitute a natural reservoir of bioactive peptides with physiological and / or antimicrobial properties, the release of which requires

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hydrolysis of the precursor molecules by digestive proteases or by fermentation with proteolytic micro-organisms. Due to safety issues and consumer demand, research into sourcing antioxidants from natural sources has been ongoing (Pihlanto *et al*., 1998). Several food protein hydrolysates have been found to exhibit antioxidant activity (Chen *et al*., 1995), (Chen *et al*., 1998), (Davalos *et al*., 2004) and (Saiga *et al*., 2003). Hence, casein hydrolysates and CPPs that retain the amino acid domain, or sequence, with antioxidant activity, could be a better option to prevent rancidity in foods without affecting other food quality parameters. Understanding the relationship between peptide composition and antioxidant activity could lead to the development of a new class of extremely effective, multi-functional antioxidants that could be used in many food applications such as the development of functional foods fortified with unstable, unsaturated fatty acids (Elias *et al*., 2008). Antimicrobial peptides produced by a wide variety of unicellular or multicellular living organisms as a first-line defense against invading micro-organisms have attracted increased attention since the discovery of lysozyme (Fleming, 1922). Many of these small molecules (<10 kDa; 3-50 amino acid residues) have proven to be potent antimicrobial substances with promising applications in medicine or food preservation.

Depending on the digestive or microbial proteases used, an array of bioactive peptides would be released either from caseins which constitute 80% of milk protein and recognized for its excellent amino acid content, slow digestion and anti-catabolic effect or whey proteins, but only a small part of these peptides has so far been identified and characterized with respect to their antimicrobial activity. The antimicrobial peptides known thus far have proven to be potent inhibitors to the growth of a wide range of undesirable micro-organisms of health or spoilage significance. Nevertheless, previous research works have largely been oriented towards their possible application in medicine, which has hindered their high potential as food-grade biopreservatives and/or as supplements in functional foods. Now, however, there is a growing interest in the use of these antimicrobial peptides as food-grade biopreservatives or as health-promoting food supplements. Such a trend is encouraged by the preference of consumers for lightly processed foods containing the least possible chemical additives and the search for functional foods perceived as nutritious, healthy and prophylactic.

Benkerroum (2008) discussed their possible application in the food industry and their mechanism of action.

Researches have been carried out on the preservative effect of lactoferrin and casein peptide; however no detailed report was published on their antimicrobial effect on pork meat and their mechanism of action on glucose metabolism in meat during storage at 4°C.

MATERIALS AND METHODS

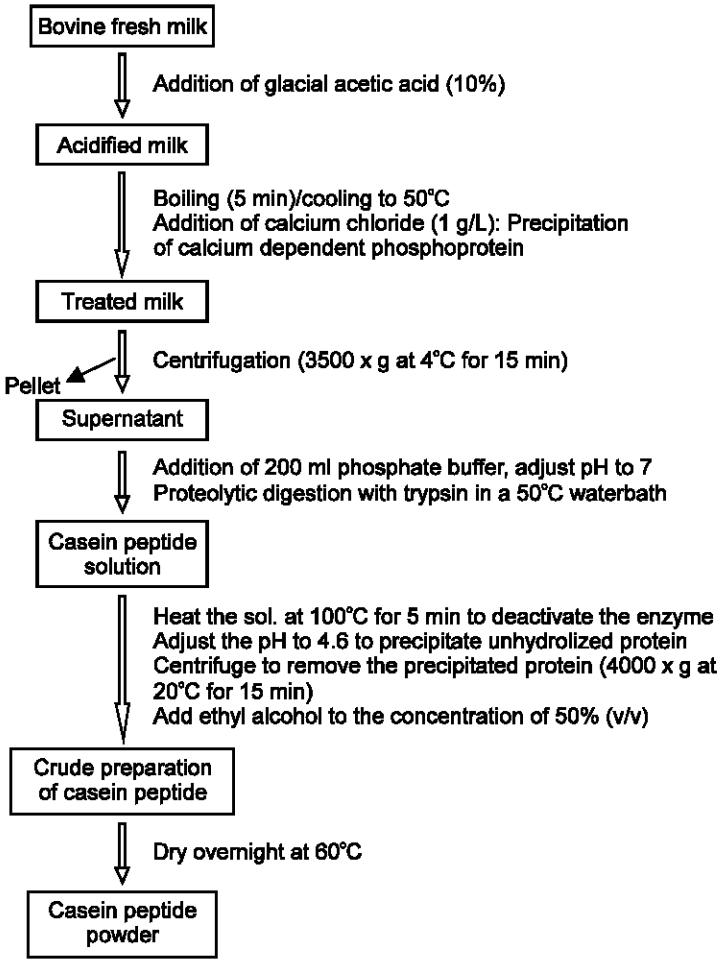
Bovine lactoferrin powder of 95.5% protein, 4% moisture and 0.5% ash was obtained from Shanghai Toang Xuean Food Technology Co. LTD., Casein peptides were prepared from milk bought at the supermarket by hydrolysis with trypsin enzyme obtained from Sigma-Aldrich (Trading Co. Ltd, Shanghai, China) together with acetonitrile and they were used without further purification. Meat was obtained from the local market; many consumers prefer to purchase hot-boned pork meat in the local markets even though chilled pork meats are available in the supermarkets. Centrifuge 5804 R (Eppendorf AG, Hamburg, Germany) was used for all centrifugation steps. An HP 1050 isocratic pump, HP 1037A refractive index detector thermostated at 40°C, HP 3396A integrator (Hewlett-Packard, Waldbronn, FRG), an MSI 660 autosampler (Kontron, Mfinchen, FRG) and an HPLC column heater (Bio-Rad) were used.

Casein peptide preparation: Casein peptides were prepared according to the method described by Benkerroum (2008) with some modification.

Meat sample preparation for glucose detection by HPLC: Boneless loins were then wrapped with Polyvinyl Chloride (PVC) film and transported to our laboratory and ground (model 4612, Hobart, Troy, OH) through a 1.6-cm plate (4-5 h after death). The meat was divided into three 1-g batches. The first batch was the control, to the second batch was added 10 mg lactoferrin and to the third 10 mg casein peptide then mixed thoroughly. For HPLC analysis, meat samples (control, with added lactoferrin and casein peptide) were prepared according to the method described by Farber and Idziak (1982). One gram portion was extensively ground in 6 mL of 6% (w/v) perchloric acid. The liquid perchloric acid fraction was decanted and centrifuged (12,100 x g; 15 min). The supernatant fraction was placed on ice, the pH was adjusted to 5.0 with 20% (w/v) Potassium Hydroxide (KOH) and the precipitated Potassium perchlorate ($KClO_4$) was removed by centrifugation at 3,020 x g for 10 min.

HPLC analysis

Chromatographic conditions: The column used was Nucleosil-NH2 (5 μm) 125 mm x 4.6 mm Internal Diameter (ID), guard column 20 mm x 4.6 mm ID (Groin, Ammerbuch, FRG). The eluent was acetonitrile/water (75+25) and the flow rate 1 ml/min at room temperature. The samples were stored at -20°C before analysis. For the standard solution 10 μL were injected onto the HPLC column and for the sample 20 μL were injected.



Method of casein peptide preparation

Total bacterial counts: At the end of each storage interval, a 25-g ground meat sample was removed and thoroughly mixed with 225-mL sterile water containing 0.1% peptone. The mixture was homogenized for 1 min at room temperature. Appropriate serial dilutions were made with sterile peptone (0.1%) water and 0.1 mL of each dilution was spread on plate count agar for total plate count (Messer *et al*., 1978). All plates were incubated at 35°C for 48 h. Colony forming units (cfu) per gram were counted.

Statistical analysis: The data were subjected to Analysis of Variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($p<0.05$) using SAS (Version 8.1, 2000; SAS Inst., Cary, NC, USA). Values expressed are means ± standard deviation.

RESULTS AND DISCUSSION

Casein peptide preparation: To reduce the cost and labour of production, it may be possible to envisage the use of crude milk-based casein peptide preparations instead of those highly purified as is required for pharmaceutical uses. Crude preparations from natural products, such as milk, do not raise food safety concerns and may thus be easily approved by health regulatory authorities for use in foods. Microgard™, lactoferrin and lactoferricins are examples of such crude preparations that have been legally approved for use in the food industry in many countries (Al-Zoreki *et al*., 1991; Tomita *et al*., 2002).

HPLC analysis: Many researchers have been conducted to assess the antimicrobial effect of lactoferrin in meat but none was done to assess its effect on glucose metabolism in meat which is of a great impact on meat freshness. Casein peptide powder was also prepared and its effect on pork meat glucose was studied. Results showed that in sample with added lactoferrin and casein peptide, glucose content increase with storage time while in pork meat sample while in the control sample, glucose was found to decrease with

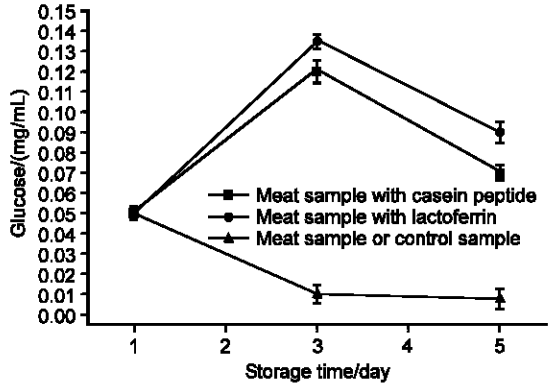


Fig. 1: Changes in glucose content in meat samples: Control sample, sample with added lactoferrin and sample with added casein peptide during storage

storage (Fig. 1). From these results it was found that lactoferrin and casein peptide have not only anti bacteria effect in pork meat but also they have an effect on glucose content in meat sample which was found to increase with added lactoferrin from 0.05 mg/mL the first day to 0.135 mg/mL the third day and then decreased to 0.09 mg/mL the fifth day and with added casein peptide it also increased from 0.05 mg/mL the first day to 0.12 mg/mL the third day and then increased to 0.07 the fifth day while for control sample it was found to decrease during storage from 0.05 mg/mL the first day to 0.01 mg/mL the third day and 0.0075 mg/mL. This can be explained by the fact that microorganisms show extreme versatility as far as the utilization of carbon source is concerned. Each of these would require a pathway that will yield essential metabolites. For example, many bacteria can grow on L-malate, succinate or glycerol but they need to synthesize hexoses for cell wall mucopeptides and storage glycogen and as carbohydrate source for nucleic acid and glycoprotein synthesis (Srivatava, 2004). During storage some bacteria need glucose for their growth; due to high concentration of amino acids such as alanine and glutamine in lactoferrin and casein peptide, glucose was synthesized by bacteria through gluconeogenesis which is the metabolic pathway where glucose is synthesized from noncarbohydrate materials when depleted. Bacteria may use gluconeogenesis to synthesize glucose from nonsugar C_2 or C_3 compounds or the intermediates of the Tricarboxylic Acid (TCA) cycle when there is not sufficient hexoses in their niches (Inui *et al*., 1999; Oh *et al*., 2002; Osteras *et al*., 1997). The first step of gluconeogenesis in bacteria is the synthesis of Phosphoenolpyruvate (PEP) through the Phosphoenolpyruvate carboxykinase (PckA) route and/or the malic enzyme-phosphoenolpyruvate synthase (PpsA) route (Hansen and Juni, 1975). The noncarbohydrate starting materials for gluconeogenesis are: lactate (from red blood cells), glycerol (from triacylglycerol hydrolysis) and certain amino acids (from muscle protein) especially alanine and glutamine which was found to be in considerable amount in bovine lactoferrin and casein protein (Castellino *et al*., 1970). Paramithiotis *et al*. (2009) reported that there are three classes of substances that are utilized by spoilage microbiota: compounds involved in the glycolytic pathway (e.g. glucose, glucose-6-P); metabolic products (e.g. lactate); nitrogen energy sources (e.g. amino acids, proteins). When the supply of simple carbohydrates or primary source of energy has been exhausted, recognizable off-odours developed leading to what is known as 'sensory' spoilage (Jackson *et al*., 1997; Stanbridge and Davies, 1998). The development of off-odours is dependent upon the extent to which free amino acid utilization has occurred (Ellis and Goodacre, 2001). The glucose content increase was one the mechanism of action of lactoferrin and casein peptide preparation to increase meat shelf-life.

Total bacterial counts: Total plate counts in hot-boned ground pork were reduced ($p<0.05$) with the addition of 80 mg lactoferrin and 10 mg casein peptide/kg at 1, 3 and 5 days of storage (Fig. 2). This suggested that the addition of lactoferrin and casein peptide inhibited microbial growth. A number of bioactive peptides have been identified in milk proteins, such as casein and whey proteins, where they are present in an encrypted form, stored as propeptides or mature C-terminal peptides that are only released upon proteolysis (Gobbetti *et al*., 2002). The first antimicrobial peptides of casein origin were identified by Hill *et al*. (1974), who isolated antibacterial glycopeptides, known as casecidins. Reduced microbial growth has been attributed to the ability of lactoferrin to sequester free iron required for microbial growth (Oram and Reiter, 1968) and casein peptides antioxidative effect.

Figure 3 show the samples incubated at the first day of storage after incubation at 35°C for 48 h. From right to left, the control sample, sample with added casein peptide and last; sample with added lactoferrin.

The results showed that prepared casein peptide powder had the effect of slowing down the microbial growth in pork meat stored at 4°C. Bovine lactoferrin powder was found to suppress the number of microorganisms in pork meat; it was highly active as it can be shown in Fig. 3.

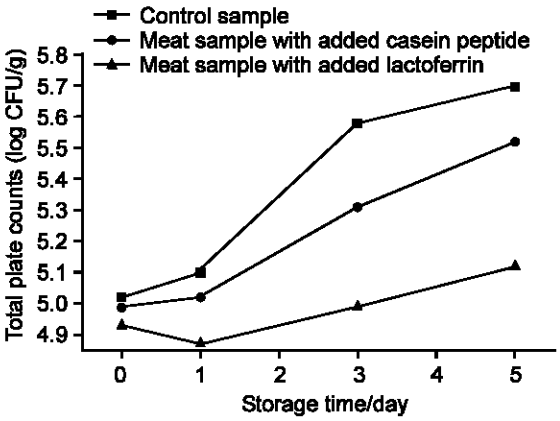


Fig. 2: Total plate counts in control sample, sample with added casein peptide and sample with added lactoferrin during storage at 0, 1, 3 and 5 day at 4°C

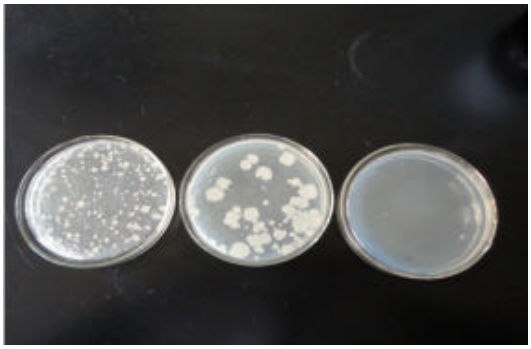


Fig. 3: Bacterial growth on plate count agar. From right to left, the control sample, sample with added casein peptide and last; sample with added lactoferrin

Practical applications: Fresh ground pork with the addition lactoferrin and casein peptide had lower total plate counts and higher glucose content. These results suggested that lactoferrin and casein peptide could be used to extend the shelf-life of various meat patties or other meat products.

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