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Purifying, Characterizing and Evaluation of Antioxidant Efficacy of Goat Milk Lactoferrin

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Abstract: The present study included isolation, extraction, and purification of lactoferrin protein from goat milk whey using AKTA Pure 25 and separation column Superdex 200 10/300 GL. A single peak appeared on 280 nm and at a flow rate of 0.5 ml/min. This peak was collected inside test tubes and kept at a 4°. The samples were submitted to electrophoresis technique by using polyacrylamide gel and the presence of dirtied materials (SDS-PAGM), one band was obtained with molecular weight reached to 75.20 Kd, while the concentration of purified Lactoferrin was 0.67 mg/ml with 6.74% carbohydrate 9.4% and 168 ppm iron. The highest antioxidant efficacy of purified lactoferrin was 81.76% at 25 mg/ml concentration, whereas α -Tocopherol reported 67.24% antioxidant efficacy at the same concentration. All concentrations of purified lactoferrin showed significant anti-oxidant efficacy in comparison with other concentrations of the natural antioxidant α -Tocopherol. BHT (industrial antioxidant) in all concentrations was superior to both lactoferrin and α -Tocopherol and recorded 95.23% at the same concentration (25 mg/ml). The purified lactoferrin portent of the goat milk whey was significant as an anti-oxidant in comparison with α -Tocopherol at all the concentrations.

Keywords: Antioxidants, Lactoferrin Goat milk, Lactoferrin

Goat's milk proteins have gained increasing attention, especially of the proteins and peptides that hydrolysis and bioactive, including lactoferrin. The interest in bioactive of goat's milk is intensifying due to its reduced allergenicity compared to bovine milk and is approaching from human milk, because it does not contain on β -lactoglobulin protein which is considered the first responsible of allergenicity of milk in children (Ahmed et al 2015). In addition to goat milk contain on an important vitamins and minerals like vitamin C, therefore it is a good source of food. The cow's milk its acidity develops and curd in 48 hours, while goat's milk needs to longer time to curd, because goat milk contains on anticoagulant compounds, including lactoferrin. The use of natural resources is safer to maintain the public health, which has made researchers and specialists to move towards their use and avoid chemicals in treating diseases, due to secondary complications and side effects of these substances. Lactoferrin protein is one of the materials naturally present in many secretions and natural fluids, which plays a fundamental role in supporting the immune system through its ability to bind iron and prevent the formation of free radicals (Al-Hatim 2012)

Lactoferrin is one of the basic milk whey glycoprotein and play a major role in the inhibition of fats and oils peroxides. The hydrolysis peptides of lactoferrin such as lactoferrin, lactoferrin and lactoferrin, has a major role to combat many diseases such cancers, inflammation, and

allergies, due to their high vital effectiveness in hindering the growth of microorganisms and inhibiting peroxides (Kanwar et al 2015). The quantities of whey that are released as waste during the dairy industry are dangerous pollutants for public health and environmental pollution and growth of microorganisms on them. In addition to these has a high nutritional value because they contain essential amino acids for human body and therefore are considered of economic importance. In world about 145 million tons annually from the whey and 30261.6 tons from Iraq which is dumped as a waste during the manufacture of cheese. Therefore, developed countries have introduced them to the food industries, such as fortifying baby food, making pastries, pharmaceutical industry, cosmetics, and making biofilms that encapsulate and support some food products, such as biofilms made from whey proteins. (Aziz 2001). The current study aimed to isolate and purified lactoferrin from goat milk whey and study the antioxidant efficacy of the isolated lactoferrin.

MATERIAL AND METHODS

The fat and caseins were isolated from goat milk to gain the whey, according to the method of Levieux et al (2006) and then dialysis was implemented by dialysis bags with molecular weight between 5-10 Kd. Freeze drying was conducted to concentrate whey samples and kept at 4° until use. Gel filtration for purification of lactoferrin protein from

goat milk whey was conducted by using ÄKTA Pure 25 and separation Colum Superdex 200 10/300 GL at flow rate 0.5 mL/min., under 1.5 MPa pressure and 280 nm wavelength. Each peak was collected by the F9-R at 2 mL/part. All samples were submitted to electrophoresis technique by using polyacrylamide gel and the presence of dirtied materials (SDS-PAGEM) for detection of protein purification and molecular weight for lactoferrin.(AL-Taai 2017). The carbohydrate content was estimated according to the method of Dubois et al (1956) and protein content was estimated as described by Lowry et al (1951). Lee and Clydesdale method (1979) was used to estimate the total iron of the purified lactoferrin protein.

Estimation of antioxidant efficacy: According to the procedure provided by Osawa and Namiki (1981) the antioxidant effect of the ferric thiocyanate was measured with some modifications and the method included mixing of an 8 mL mixture of the buffer phosphate 0.05M and pH 7 with 4.1mL of linoleic acid at 2.5% concentration ethanol with the addition of 4 mL of lactoferrin protein and 3.9 ml distilled water. Then the mixture was incubated at 40 ° for 24 hr. then and 0.1 ml of the incubated mixture was added to 9.7 ml ethanol with a concentration of 75% and 0.1 ammonium thiocyanate with a concentration of 30% and 0.1 of ferrous chloride of 0.02 M (0.07 g in 20 ml of hydrochloric acid with a concentration of 3.5%) and then shake gently. The antioxidant efficacy was measured at a wavelength of 500nm.

$$\text{Inhibition} = \left\{ 1 - \frac{(\text{Abs control})}{(\text{Abs model})} \right\} \times 100 \quad \text{Abs : Absorption}$$

Samples of the control group were prepared in the same way above, by replacing protein sample with 4 mL ethanol. The purified samples of lactoferrin have been compared with BHT, α -Tocopherol and control group at a concentration ranging from 5-25 mg/ml.

RESULTS AND DISCUSSION

The fatty layer of goat milk was isolated at a speed of 4500 - 5,000 rpm for 30 minutes using a refrigerated centrifuge. The speed was then increased to 10,000 - 20,000 rpm for 20 minutes after the addition of diluted HCL droplets until reaching to electric point 4.3 to separate small, medium and large casein of goat milk and the goat milk whey was obtained. Then partial purification was carried out by using a dialysis bags. The result agree with AL-Hatim (2012).

Ionic exchange: The results of ion exchange chromatography which has been representing the most common method used for purifying milk proteins, showed the ion exchange of goat milk whey along the 280 nm wavelength at a 3-3.5 ml/min flow rate. The first peak representing the

lactoperoxidase protein at 0.2 M salt gradients of NaCl. The second peak with the pink-color represented lactoferrin protein, the pink color attributed to the conjugation of lactoferrin to iron ions and the highest absorption point was 0.49 along the wavelength of 465 nm (Fig. 1). These findings are consistent with results of Abbas (2015).

Gel filtration to purify lactoferrin: The purification of the lactoferrin protein from the goat milk whey after the ion exchange phase, the second isolated peak was taken from the ion exchange phase and injected in the ÄKTA pure 25 on the supersdex 200 10/300 GL. Isolation conditions were fixed on the 280nm wavelength at 0.5 mL/min and 1.5-2 MPa pressure using the basic tris-HCL with pH 8.7 and 0.02 m concentration to get the whole purification of the isolated parts of the goat's milk whey, one peak with a maximum absorption of 191 mAU was appeared at 14.34 minutes (Fig. 2) and these results are in accordance with Abbas (2015) and AL-Rikabi et al (2016).

Electrophoresis with presence of dirtied materials (SDS-PAGM): The results of electrophoresis by using polyacrylamide gel and the presence of materials (SDS-PAGM) for the purified lactoferrin protein in the ion exchange and gel filtration to determine the molecular weight of lactoferrin goat milk whey in comparison with standard proteins after calculation of the proportional movement. One band was obtained with molecular weight of 75.20 Kd (Fig. 3) which indicates that the protein has reached a high purity level and these results are agree with Abbs(2015) and Alluwaimi et al (2017).

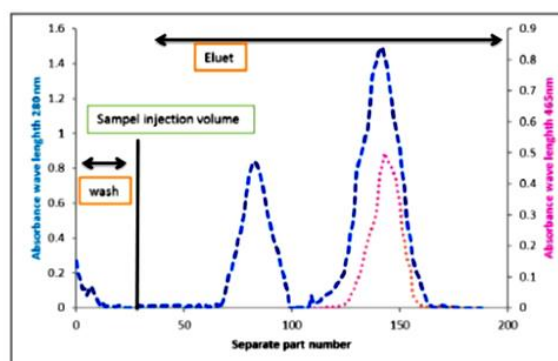


Fig. 1. Chromatography ionic exchange of goat milk whey using cation ionic exchange cm-sephadex C-25, dimension 1.5 x 60 cm, At flow rate of 3.5 ml/min, the column was balanced with the Tris-HCL solution of 0.05 m and pH 7.8. The first phase of the recovery (ELUTE) was represented by using of the tris-HCL of 0.05 m, PH 7.8 which was contain NaCl of 0.2 m and 0.5 m of NaCl was used in the second phase of recovery

Lactoferrin concentration: Dialysis bags were used for 24 hr. in two phases which have molecular weights of 5,000 Kd and 7 Kd for partial purification of whey proteins, and buffer solution of the Tris-HCL with pH 8.6 and 0.02 M for maintaining the pH of Lactoferrin. Then all samples were submitted to freeze drying until reaching to 0.68 mg/ml after the ion exchange phase in lactoferrin of goat milk whey and 0.83 mg/ml at the end of the purification phase, as its shown in Table 2 (Hiss et al 2008).

Content of lactoferrin carbohydrates: The content of lactoferrin carbohydrates was 6.74% and these results were consistent with the Shimazaki (2000) and observed that carbohydrate of cows lactoferrin ranged between 7-11.5%.

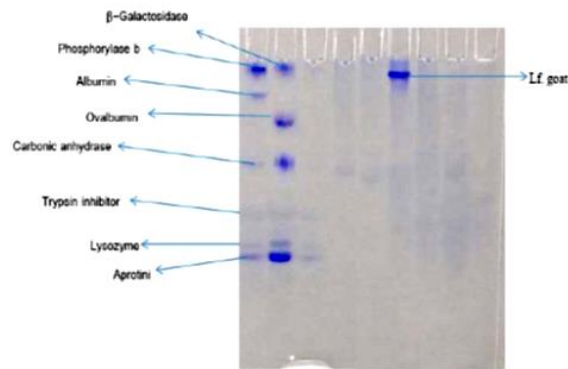


Fig. 2. Gel filtration for the second peak of the ionic exchange of goat milk whey using the Äkta Pure 25, on the Superdex 200 10/300 GL at flow rate of 0.5 ml/min on 280 nm

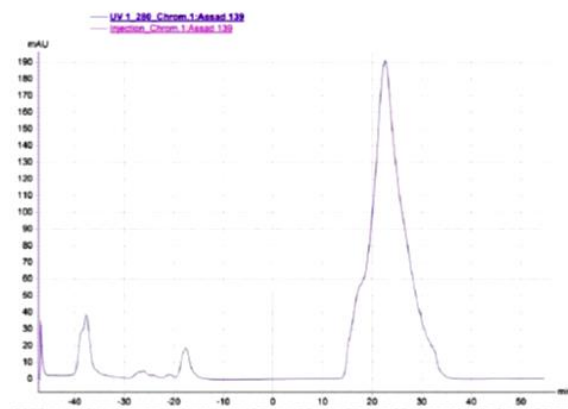


Fig. 3. Shows the Electrophoresis by using polyacrylamide gel and the presence of dirtied materials(SDS-PAGM) and 2-mercaptoethanol to estimate molecular weight and confirm the purity of lactoferrin goat milk whey at 12% concentration

Total iron content of lactoferrin protein: The level of saturated iron in purified lactoferrin protein of goat milk whey was 9.4% and 168 ppm (Table 3) and this result is due to the high content of carbohydrate for lactoferrin protein of goat milk. This indicated a greater potential of lactoferrin protein for binding free iron in the center especially with the greater openness in C lobes of protein (Baker & Baker 2005). These results are in line with Shimazaki (2000), who found observed that the rate of saturated iron in the camels lactoferrin was 9%. Aziz said (2001) reported that the contents of the saturated iron in cows and buffalos lactoferrin are 13.85 and 18.98%, respectively.

Antioxidant efficiency: High antioxidant effectiveness of lactoferrin of goat milk whey for linoleic acid peroxides was 81.76% at 25 mg/mL (Fig. 4). Earlier workers also observed similar trend (Simos et al 2011, Habib et al 2013, Ahmed et al 2015). This variation are in the anti-oxidant effect to protein lactoferrin are due to different sources, materials, and purification devices, as well as a variety in the quantity and quality of protein in addition to the differences of molecular weight and the number and locations of amino acids involved in protein structure.

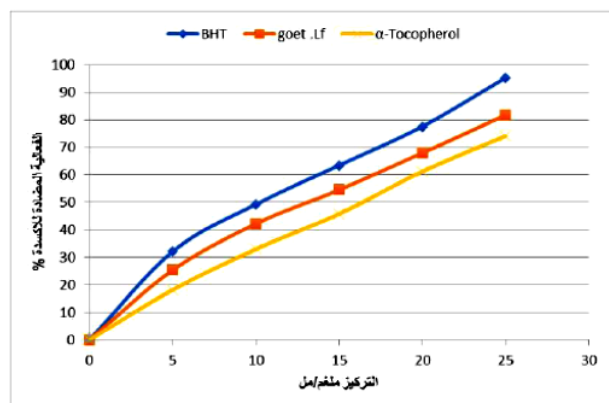
The highest anti-oxidant effect for linoleic acid peroxides 95.23% at 25 mg/ml was in BHT group, while the highest anti-oxidant effect of α -Tocopherol was 74.27% at the same concentration. These findings are close to those of the Al-Hatim (2012). The statistical analysis showed significant increase in the antioxidant of the industrial BHT in all concentrations in comparison to lactoferrin and α -Tocopherol. The lactoferrin exhibited high significant antioxidant effect as compared to α -Tocopherol. The highest antioxidant effect of lactoferrin of goat milk whey is due to the high capacity to give hydrogen electrons and binding iron ions with lactoferrin leading to chelate the free radicals in addition to the differences of the number and locations of

Table 1. Standard proteins used in electrophoresis technique by polyacrylamide gel and the presence of dirtied materials (SDS-PAGM)

| Source | Molecular weight (kilo dalton) | Standard protein name |
|---------------------|--------------------------------|------------------------|
| <i>E. coli</i> | 116 | β -Galactosidase |
| Rabbit muscle | 97.4 | Phosphorylase b |
| Bovine serum | 66 | Albumin |
| Chicken egg white | 45 | Ovalbumin |
| Bovine erythrocytes | 29 | Carbonic anhydrase |
| Soybean | 20.1 | Trypsin inhibitor |
| Chicken egg white | 14.4 | Lysozyme |
| Bovine lung | 6.5 | Aprotinin |

Table 2. Concentration of protein lactoferrin purified of goat milk whey and content iron

| Stages of the treatment of the lactoferrin | Goat |
|--|----------------------------|
| After the ion exchange | 0.68 Mg/ml |
| After gel filtration | 0.83 Mg/ml |
| Total iron content | Part in % 9.4, 168 million |
| Carbohydrates | 6.74% |

**Fig 4.** Antioxidant efficacy of a lactoferrin protein debuster compared to the BHT and α -tocopherol

amino acids involved in proteins molecule structure as well as the differences in the number and locations of hydroxyl groups in the amino acids structure (Pihlanto 2006). The antioxidant effect of lactoferrin of goat milk whey compared with the normal and industrial antioxidants might be attributed to the difference in the material, purification methods and type of solvent used for isolation, thus lactoferrin possess properties depends on molecular structure and presence of hydroxyl and ketone groups in half of each rings with the reciprocal double-bonds.

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