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Isolation, Purification and Characterization of Lactoferrin from Goat Colostrum Whey

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Abstract: Lactoferrin is an important protein in many biological applications as a potential cancer treatment agent. In this study, lactoferrin was purified from goat colostrum by ion exchange chromatography through CM-Sephadex C-50 column and gel filtration chromatography through Sephadex G-200 column. The purification fold and yield were (20.83 time and 62.50%) respectively. The purity of lactoferrin to homogeneity was examined by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) with single bond. Some biochemical characteristics of the purified Lactoferrin were determined, The molecular weight of LF were 80 and 79.50kDa as determined by gel filtration and SDS-PAGE respectively . The percentage of carbohydrate content in goat Lactoferrin was 10.4%, mean while the Iron percentage was 123 ppm and as a saturated percent 7.8%.

Key words: Lactoferrin, goat colostrum, isolation, purification, characterization

INTRODUCTION

Milk and dairy products have become recognized as functional foods, suggesting their use has a direct and measurable effect on health outcomes, namely that their consumption has been related with a reduced risk of numerous cancers (Marshall, 2004; Esmat *et al*., 2013). Milk proteins Classified upon their concentration to two main groups first group called the main proteins which include Casines (alpha, beta and kappa casein) and two type of whey proteins called a-lactalbumin and B-lactoglobulin, The second group called secondary or (minor) proteins which include Lactoferrin, immunoglobulin, and two enzymes called Lysozyme and lactoperoxidase which whey proteins recognized as the main source of it (McSweeney and Fox, 2013). In spite of the fact that the second group of proteins constitute a small percentage of the total milk proteins, but it plays a big role (directly or indirectly) as the first lines of defense because of their great ability to work as antimicrobial, antioxidant and anti-cancer (Korhonen and Pihlanto, 2006; Rodrigues *et al*., 2009). Lactoferrin (LF) was received considerable attention in recent years, to being one of the biologically active compounds which possess actually role as anti-microbial, immune modulator, anti-inflammatory, anti-oxidant and anti-cancer (Tsuda *et al*., 2010; Pan *et al*., 2007; Rodrigues *et al*., 2009). LF is a glycoprotein with a molecular weight of about 80 kDa. Due to the size and construction, it belongs to the transferrin family, which has a specific ability to bind iron (Legrand *et al*., 2008). It occurs as a single polypeptide chain which consists of about 690 amino acids (Baker and Barker, 2005). LF is an iron-binding glycoprotein from the transferrin family, LF found in many different tissues or secretions, such as tears, saliva, blood, secondary granules of neutrophils and milk (Pan *et al*., 2007; Rodrigues *et al*., 2009). *In vivo* studies showed that oral administration of bovine LF to rodents significantly reduces chemically induced tumorigenesis in different organs (breast, esophagus, tongue, lung, liver, colon and bladder) and inhibits angiogenesis (Tsuda *et al*., 2010). There is no study in Iraq about the Isolation and purification of Lactoferrin from goat colostrum there for this study was done by using ion exchange chromatography and gel filtration also some of its characters, (Molecular weight, Carbohydrate content and Iron content) was studied.

MATERIALS AND METHODS

Goat colostrums: Goat colostrum was obtained from Ruminants researches station, Directorate for Agricultural Researches - Ministry of Agriculture, Abu-Grip-Baghdad. The samples were collected within the first five days after goat parturition and were immediately frozen and stored at -18°C until use. The colostrum was skimmed by centrifugation in a Sigma MA3-18 centrifuge at 4000 g/min for 30 min at 4°C. Colostrum whey was prepared by precipitation of the casein from skimmed colostrum in acidic condition with gradual addition from 1N HCl until pH reached to 4.6, the precipitated casein was removed by centrifugation at 10000g/min for 15 min at 4°C. The supernatant (whey) was adjusted to pH 6.8 with 1N NaOH and dialyzed against distill water for 18 hr, and then stored at -18°C until use. (Al-Mashakhi and Nakai, 1987).

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Isolation and purification of lactoferrin: Isolation and purification procedures by Yoshida *et al.* (2000) were used. The procedure involved cation exchange chromatography (CEC) using cation exchanger carboxymethyl Sephadex-C50 (CM-Sephadex C-50) and gel filtration chromatography by using Sephadex G-200.

Protein determination: Bradford (1976) methods was used for protein determination.

Purity test: Done by using polyacrylamide gel electrophoresis under denaturated condition (SDS-PAGE) method by Laemmli (1970).

Molecular weight Determination by electrophoresis (SDS-PAGE) : Method described by (Weber and Osborn, 1969) was used. Standard protein with a molecular weight ranging from 15000 to 170000 Dalton was also loaded. Lactoferrin molecular weight was calculated from drawing the relationship between the logarithms of the molecular weight for standard proteins compared to relative mobility.

Carbohydrate content: Phenol-Sulphuric acid procedure was used in determined carbohydrate in lactoferrin (Dubois *et al.*, 1956).

Iron content: Total Iron content was estimated according to Lee and Clydesdale (1979) method. Total iron concentration in purified Lactoferrin calculated as a fallow:

Total iron concentration (ppm) = (Atomic absorption for sample - Atomic absorption for blank) x final sample volume / sample weight (gm) x dilution factor

RESULTS AND DISCUSSION

Isolation and purification of lactoferrin: Two steps were used to purify Lactoferrin from colostrum whey, ion exchange chromatography and gel filtration were applied respectively.

Ion-exchange chromatography: CM-Sephadex C-50 cation exchanger was used in goat milk Lactoferrin purification due to its important properties such as easy of preparation and the possibility of reused after reactivated (Bonner, 2007). Whey colostrum which prepared above was pass through CM-Sephadex C-50 column, 0.05M of Tris HCl buffer pH 7.5 solution was used as equilibration buffer, Fig. 1 shows one protein peak appeared in the washing step belong to unbounded proteins like alpha-Lactalbumin and beta-Lactoglobulin, while two protein peaks appeared in the elution the first protein peak with green color refers to the lactoperoxidase enzyme, while the second protein peak was given pink color which shows high value when read on the wave length of 465nm Special detects for protein Lactoferrin (Fig. 2) (Shimazaki *et al.*,1992). Results indicated that the first peak in elution appeared in fraction numbers (91 to 110) and the second peak which has LF that appeared in fraction (165-175). The first peaks eluted at 0.2M of NaCL, mean while the second peak eluted by using 0.5M of NaCl. The fractions of second peak were pooled and desalted by dialyzing against distilled water overnight, and then concentrated by sucrose.

Fig. 4 reveals that SDS-PAGE for purified LF from this step was a homogenous preparation with an identical migration pattern as the standard bovine Lactoferrin obtained from Sigma (lanes 2). Protein content of each fraction was also determined by Bradford method, the concentration of LF from this step of purification as determined by Bradford assay was about 300 mg/1L. The results of this study compatible with several other studies Nam *et al.* (1999) purified LF from goat milk by using CM-Toyopearl 650M column followed with AF-Heparin Toyopearl column, and they found two protein peaks in elution the first peak belonged to lactoperoxidase enzyme and the second peak belonged to LF. Whereas Fee and Chand (2006) used ion exchange chromatography using SP-Sepharose column to purify lactoperoxidase and LF from cow milk, two peaks in eluted step was found the first with green color and the second with pink color. The results resemble those found by Lu *et al.* (2007) which purified lactoperoxidase and LF from cow milk, by SP-Sepharose column, while Wolman *et al.*, (2007) used one step by affinity membrane chromatography when purified Lactoferrin from bovine whey and colostrums. Angeles *et al.*, (2008) isolated LF from Carabo's *(Bubalus bubalis* L.*)* milk whey by ammonium sulfate precipitation and cation exchange chromatography using carboxymethyl cellulose (CMC), Carabo LF was eluted at approximately between 0.27-0.30 M NaCl with a concentration of 20.05 mg/l and a recovery of 24.97%. Younghoon *et al.* (2009) purified LF from goat colostrum by CM-Sephadex C-50 ion-exchange column and affinity chromatography on *Hi-Trap* Heparin HP. Yafei *et al.* (2011) isolated Lactoferrin and lactoperoxidase from bovine colostrum by one-step cation exchange chromatography with SPEC 70 SLS ion-exchange resin. Moradian (2014) enabled purified LF from colostrum of cow's milk with one step by using CM-sephadex C-50, a cation exchange chromatography and they determined LF concentration by Bradford assay which was about 2.4 mg/ml, with good biological activity and purification efficiency was about 90%.

Gel filtration chromatography: After purification by ion exchange, fractions representing LF were collected and dialyzed against distill water then concentrated by sucrose for applying to gel filtration chromatography by using Sephadex G-200 column. Fig. 3 showed that one

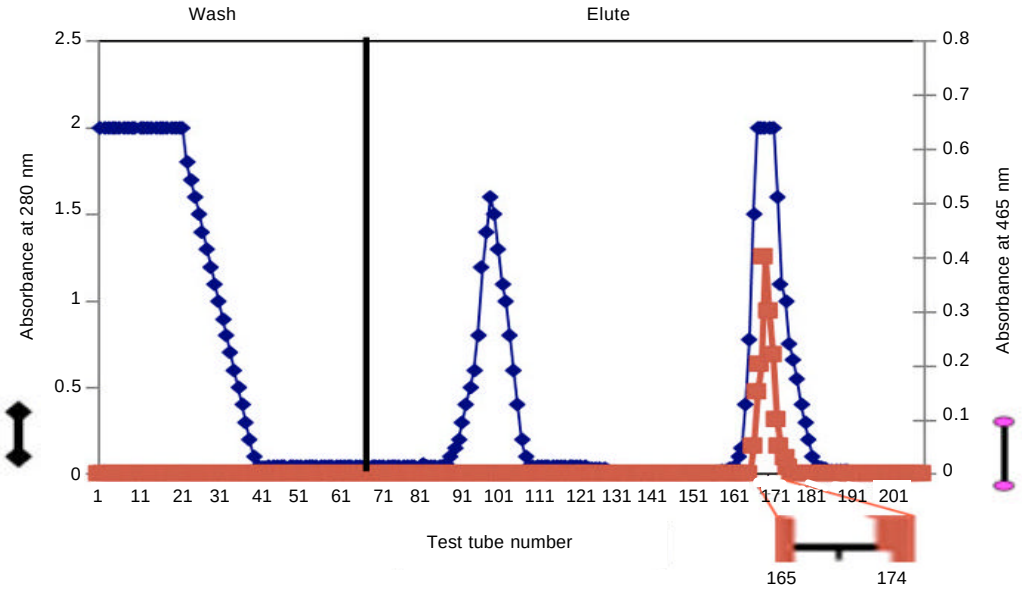

Fig. 1: Ion exchange chromatography for purification gLF by using CM-Sephadex C-50 (2×24 cm) column, equilibrated with Tris-HCL buffer (0.05M, pH7.5), eluted with Tris-HCL buffer with NaCl gradient 0.2 and 0.5 M in flow rate 18 ml/hr., 3ml for each fraction.

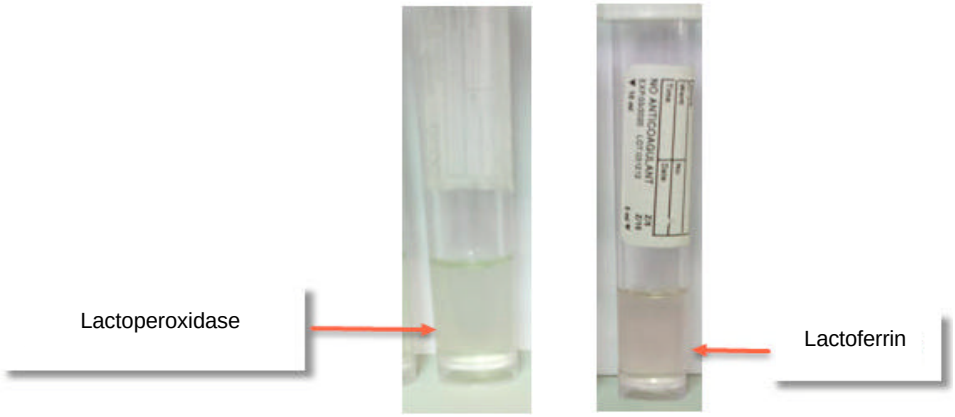

Fig. 2: The lactoperoxidase (green color) and goat milk Lactoferrin (pink color) from ion exchange chromatography.

protein peak with light pink color appeared in the eluted fractions (20 to 30) had the LF protein which shown high value when read on the wave length of 465nm Special detects for protein Lactoferrin. Gel filtration method was used widely in extra purification step after ion exchange to obtain the highest level of protein purity which was confirmed by polyacrylamide gel electrophoresis, Sephadex G-200 was used in many studies of LF purification. Legrand *et al.* (2008) purified LF from human milk by Sephadex G-200 column, while Kim *et al.* (2009) applied Sephadex G-100 column in purification of LF from mare milk. Al-Hatim (2012) used sephadex G-150 in purification of LF from Cow and Sheep milk and they found single band when SDS-PAGE used to test purity. The concentration of LF from this step of purification as determined by Bradford assay was about 250 mg/1L. Table 1 showed the concentration and recovery yield of LF at each step of the overall separation process from the data LF concentration reduced with the progressed in purification steps it was 400, 300 and 250 mg/1L for goat colostrum whey, LF from ion exchange step and LF from gel filtration step. whereas the final protein recovery and the purification number were 62.50% and 20.83 time respectively. Purified LF in the present study had a very good concentration and its purification efficiency was about 90%. The mentioned method, apart from simplicity and speed, can result in isolation of highly pure LF (Fig. 4). The results in this study were similar to those results found by Moradian (2014) which he used

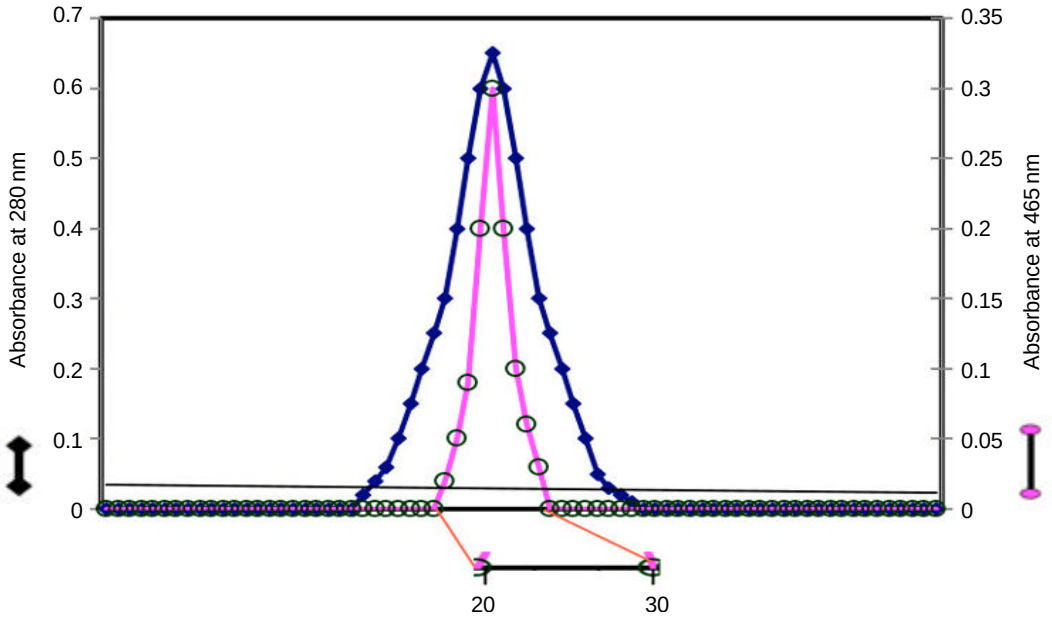

Fig. 3: Gel filtration chromatography for purification gLF by using Sephadex G-200 column (1.5 × 60 cm), equilibrated with 0.5 M phosphate buffer containing 0.01 M NaCL, pH 7.4 with a flow rate of 18 ml/hr, 3ml for each fraction .

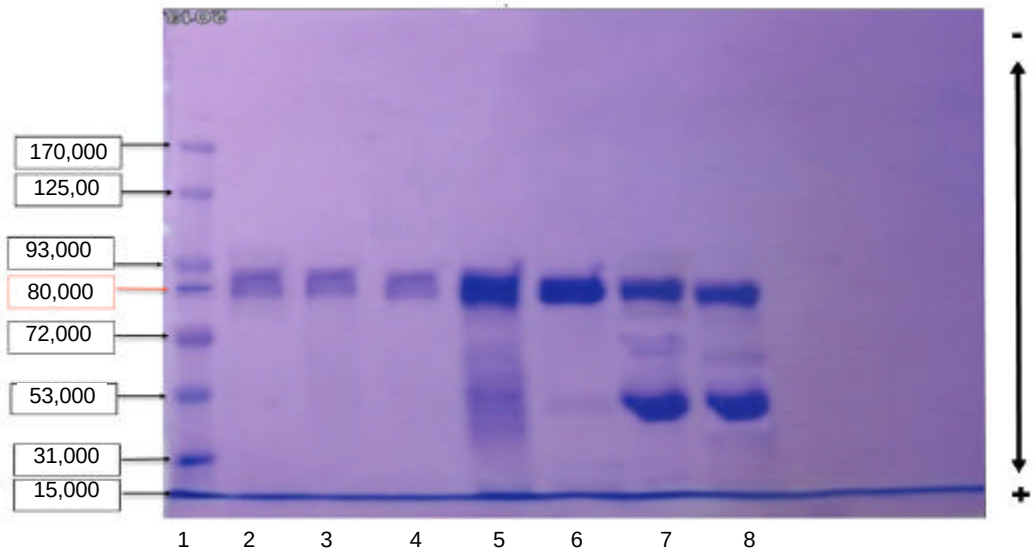

Fig. 4: Polyacrylamide gel electrophoresis of LF 7&8: Colostrum Whey, 5&6: LF after Ion exchange chromatography, 3&4: LF after gelfiltration step, 2: standard Bovine Lactoferrin, 1: Molecular weight of the protein marker from (15-170) kDa.

only one step in purified bLF with biological activity and the purification efficiency was about 90%.

Determination of lactoferrin purity by SDS-PAGE: Lactoferrin purity was detected by electrophoresis using polyacrylamide gel under reducing condition. Electrophoresis on polyacrylamide gel can be used as another step of purification and for determining the efficiency of purification steps. The protein profile of LF purified to homogeneity which gave one protein band located in the upper part of the gel (Fig. 4). This result confirmed the purity of LF isolated from colostrum whey that means the purification steps were successful in purified this protein. Younghoon *et al.* (2009) used SDS-PAGE to confirm purity of caprine LF which was purified by CM-Sephadex C-50, they found single band in 12.5% gel. Yafei *et al.* (2011) also obtained a single band in the gel of SDS-PAGE to confirm purity of isolated LF from

Table 1: Concentration and recovery yield of LF at each step of the overall separation process

	Colostrum whey	Ion-exchange step	Gel filtration step
Concentration of LF (mg/1L)	400	300	250
Recovery relative to whey solution (%)	100	75	62.50
Purification number	-----	18.75	20.83

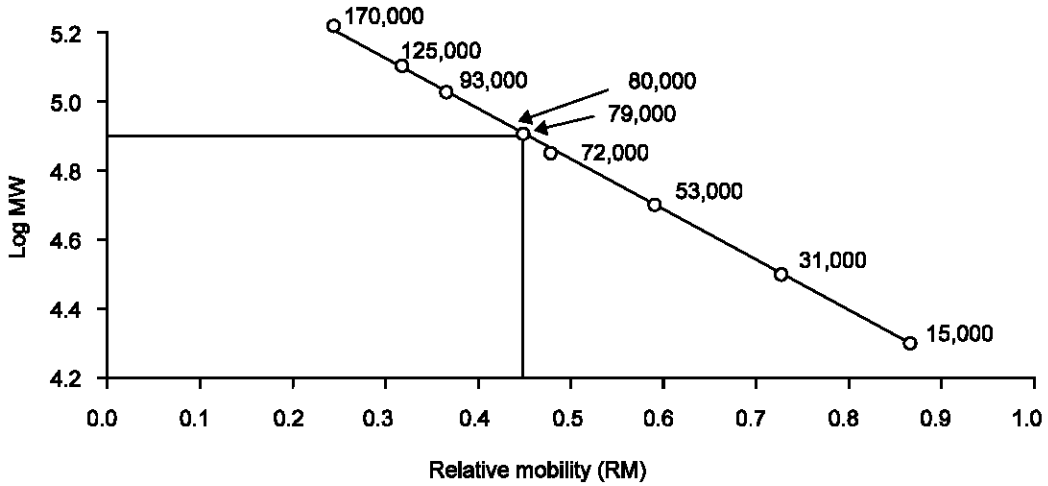

Fig. 5: Standard curve for determination LF molecular weight using SDS-PAGE

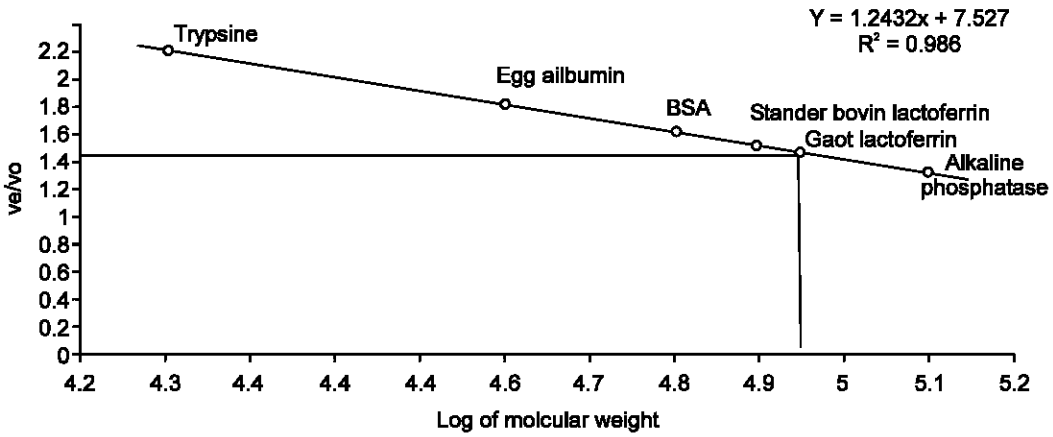

Fig. 6: Standard curve for determination LF molecular weight using gelfiltration chromatography

defatted bovine colostrum, Moradian (2014) also used SDS-PAGE to confirm LF purity which was isolated from Colostrum of cow's milk by using CM-sephadex C-50.

Characterization of the purified lactoferrin

Estimation of Lactoferrin molecular weight: Molecular weight of lactoferrin was estimated by SDS-PAGE. One protein band located in the upper part of the gel was detected, and this indicated that LF had large molecular weight. The method used by measuring the relative mobility (Rm) of LF as described in Fig. 4 and 5 depends on the (Rm) of standard proteins versus its log of molecular weight. Results showed that the approximate molecular weight of gLF was 80KDa.

Molecular weight of LF differs according to the type of animal and carbohydrate content which varies according to LF source which gave LF molecular weight greater than reality (Lambert *et al*., 2005). When comparing the result obtained of LF in this study with those of other studies it was found approach to the conclusion that found by Nam *et al*. (1999) about the M.W of LF isolated from goat milk which determined by SDS-PAGE as 82 KDa. Adam *et al*. (2008) employed SDS-PAGE in determining the molecular weight of cow milk LF which was detectable as 77 kDa. Whereas Angeles *et al*. (2008) predicated the molecular weight of carabao's LF approximately 77,829 KDa as estimated by SDS-PAGE. Younghoon *et al* . (2009) found that the molecular weight

of caprine LF was 82 KDa, Annabelle *et al.* (2014) determinate the molecular weight of goat milk LF by SDS-PAGE as 78 KDa.

Molecular weight of Lactoferrin estimated by gel filtration Gel filtration chromatography was used to estimate LF molecular weight, according to the standard curve which represented the relationship between the log of molecular weight of standard proteins versus (Ve / Vo) as illustrated in Fig. 6 which showed that the molecular weight of LF purified from goat colostrum was 80 Kda. This result was close to results found by Aziz (2001) about the molecular weight of LF isolated from cow and buffalo milk as determined by gel filtration which was 82.22 and 78.52kDa respectively. Another study by Farrel *et al.* (2004) reported a MW for bLF as 76,110 representing 708 amino acid residues, while the molecular weight of human LF was 80kDa as determined by gel filtration (Baker and Baker, 2005).

The molecular weight of LF isolated from different source ranged between 76-87 kDa (Wang and Hurley, 1998). Park *et al.* (2007) found the molecular weight of sheep milk LF was 78kDa while Castillo *et al.* (2010) concluded that the molecular weight of LF purified from cow colostrum when using Sepharose -4B column was 82kDa .

The carbohydrate content in lactoferrin: The result of carbohydrates content showed that LF protein which was isolated from Goat colostrum contains 10.4% carbohydrates. This result consisted with those found by Shimazaki (2000) about carbohydrates content in LF which ranges between 7-11.5%. The difference in the types of mammals can lead to a difference in the content of the molecule from Sialic acid or the difference in the number of sugar chains of the protein molecule (Van Veen *et al.*, 2004). Carbohydrate analysis by phenol-sulfuric acid method showed 0.450% and 0.346% carbohydrate for bLF and carabao's LF, respectively (Farrell *et al.*, 2004). Brisson *et al.* (2007) found that bLF contain 11.2% carbohydrate, hLF contains 6.4%, horse 3.5% and camel 11%. Al-Hatim (2012) found the carbohydrates content in cow and sheep milk were 11.1% and 8.2% respectively.

The iron content in Lactoferrin: The results of the assessment of iron saturation in goat milk LF by atomic absorption spectrophotometer and the iron content were 8.7% and 123 ppm respectively. The results were close to that found by Kappeler (1998) about iron saturation of camel milk LF which was 9%, while Jan and Hooijdonk (2000) reported that the natural state bLF was only partly saturated with iron (15±20%) and has a salmon pink colour, the intensity of which depends on the degree of iron saturation. Iron-depleted Lactoferrin with less than 5 % iron saturation is called apolactoferrin, whereas iron-saturated LF is referred to as hololactoferrin. In breast milk the LF found is essentially apolactoferrin. Another study was done by Ella *et al.* (2009) about hLF iron saturation which ranged from 5-15% . Rebelein (2010) refereed to the iron saturation in bLF as 15-20%, Lactoferrin belongs to the transferrin family, which has a specific ability to bind iron (Baker and Baker, 2005; Legrand *et al.*, 2008). Each LF molecule binds two Fe^{3+} ions.

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