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Kinetic and thermodynamic parameters for thermal denaturation of ovine milk lactoferrin determined by its loss of immunoreactivity

F. Navarro,[1] S. Harouna, M. Calvo, M. D. Pérez, and L. Sánchez[2]
Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, España

ABSTRACT

Lactoferrin is a protein with important biological functions that can be obtained from milk and by-products derived from the dairy industry, such as whey. Although bovine lactoferrin has been extensively studied, ovine lactoferrin is not quite as well known. In the present study, the effect of several heat treatments in 3 different media, over a temperature range from 66 to 75°C, has been studied on lactoferrin isolated from sheep milk. Denaturation of lactoferrin was determined by measuring its immunoreactivity with specific polyclonal antibodies. Kinetic and thermodynamic parameters obtained indicate that lactoferrin denatures by heat more rapidly in whey than in phosphate buffer or milk. The value of activation energy found for the denaturation process of lactoferrin when treated in whey is higher (390 kJ/mol) than that obtained in milk (194 kJ/mol) or phosphate buffer (179 kJ/mol). This indicates that a great amount of energy is necessary to start denaturation of ovine lactoferrin, probably due to the interaction of this protein with other whey proteins. The changes in the hydrophobicity of lactoferrin after heat treatments were determined by fluorescence measurement using acrylamide. The decrease in the hydrophobicity constant was very small for the treatments from 66 to 75°C, up to 20 min, which indicates that lactoferrin conformation did not experienced a great change. The results obtained in this study permit the prediction of behavior of ovine lactoferrin under several heat treatments and show that high-temperature, short-time pasteurization (72°C, 15 s) does not cause loss of its immunoreactivity and, consequently, would not affect its conformation and biological activity.

Key words: ovine lactoferrin, sheep milk, thermal denaturation, whey

INTRODUCTION

Milk contains many bioactive components, and among them, those with defensive activity are of great importance for the newborn. Lactoferrin, which is present in the milk of the majority of mammalian species, takes part in the nonspecific defensive system of this secretion, together with lysozyme and lactoperoxidase. Lactoferrin is an iron-binding protein that belongs to the non-heme transferrin family, and it has been described as a multifunctional protein, because it can exert several biological functions related to its antibacterial activity, antioxidant properties, antitumoral activity, and participation in iron absorption (Sánchez et al., 1992a). All these properties confer a great value to lactoferrin for its use as supplement in functional products or in nutraceutical products.

The procedure most widely applied to preserve milk and dairy products is heat treatment. However, heat treatment has some adverse effects in milk products because it causes a decrease in the levels of some nutrients and impairs their organoleptic characteristics (Considine et al., 2007). Furthermore, heat treatment induces denaturation and aggregation of milk proteins, thus modifying their biological activity and technological properties (Anema, 2009). The effect of heat treatment on lactoferrin has been studied in several ruminant species, such as bovine (Sánchez et al. 1992b) and caprine (Sreedhara et al., 2010), and also in human lactoferrin from milk and from recombinant origin (Mata et al., 1998; Conesa et al., 2007; Mayayo et al., 2014). Those studies have been performed by using several analytical techniques to evaluate denaturation, such as differential scanning calorimetry (Sánchez et al., 1992b; Mata et al., 1998; Conesa et al., 2007), fluorescence measurement, circular dichroism (Sreedhara et al., 2010), and immunochemical techniques (Sánchez et al., 1992b). However, as far as we know, no systematic studies have been reported on thermoresistance of ovine lactoferrin. Cow milk is normally the source selected for obtaining lactoferrin to be used as ingredient, although other materials such as whey from cheese manufacture can also be used. The production of cheese from sheep milk is very important in the Mediterranean countries

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[1]Present address: Facultad de Medicina, Escuela de Nutrición y Dietética, Universidad de Los Andes, Avenida Don Tulio, Mérida 5101, Venezuela
[2]Corresponding author: lousanchez@unizar.es

and, consequently, a large amount of whey is generated in this process. Whey is a valuable by-product due to its protein content of high level and quality, and is also a good source for bioactive proteins, such as lactoferrin or lactoperoxidase. The procedure for cheese manufacture comprises multiple technological variables related to temperature of treatment for milk pasteurization and curd heating before whey draining. Therefore, it is necessary to know the influence of those conditions when the whey is going to be used to isolate bioactive proteins.

The aim of this work has been to study the effect of various heat treatments on the denaturation process of ovine lactoferrin in 3 different media. Thus, the thermoresistance of lactoferrin in sheep milk and whey and also of isolated lactoferrin in phosphate buffer has been determined. Denaturation of lactoferrin has been estimated by measuring its immunoreactivity and determining hydrophobicity that indicates the integrity of the conformational protein structure.

MATERIALS AND METHODS

Obtaining Lactoferrin from Ovine Milk

Sheep's raw milk was donated by Villacorona (El Burgo de Ebro, Zaragoza, Spain). Milk was skimmed by centrifugation at 2,000 × *g* (4°C) for 15 min. Then, milk was coagulated with rennet from Abiasa (Pontevedra, Spain) in a proportion of 1:15,000, maintaining milk in a thermostatic bath at 34°C for 40 min. Coagulated caseins were eliminated by centrifugation at 2,000 × g (4°C) during 15 min and the whey obtained was dialyzed in a membrane with a 10-kDa molecular weight cutoff (**MWCO**), against 0.015 *M* Tris-HCl buffer, pH 6.8. The dialyzed whey was applied to a CM-Sephadex column previously equilibrated with the same buffer, at a flow rate of 0.1 mL/min, and the fractions collected were of 2 mL. To elute the bound proteins, a first elution step was applied with 0.2 *M* NaCl and a second one with 0.5 *M* NaCl in the same Tris buffer. The fractions that contained lactoferrin were concentrated in ultrafiltration cones of 10-kDa MWCO, dialyzed and lyophilized. Fractions from chromatography were analyzed by SDS-PAGE.

Obtaining Rabbit Antisera Against Ovine Lactoferrin

Antisera against ovine lactoferrin were developed in rabbits following procedures under Project License PI48/10 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes. A volume of 0.5 mL of ovine lactoferrin (2 mg/mL) was homogenized with 0.5 mL of complete Freund's adjuvant and administered in several subcutaneous injections in the back of the animals. After 3 wk, the animals were boosted following the same protocol as in the first immunization, although using incomplete Freund's adjuvant. Ten days later, the animals were cannulated and bled from an ear vein. Double immunodiffusion and immunoelectrophoresis were used to test the antisera, which were shown to be specific for ovine lactoferrin.

Thermal Treatment of Ovine Lactoferrin

For the study of the effect of heat treatment on lactoferrin denaturation, skim milk and whey were used, in which lactoferrin concentration was of 0.2 mg/mL. The thermoresistance of lactoferrin in 0.05 *M* monosodium phosphate buffer, 1 *M* NaCl, 1 *M* KCl, pH 7.4, was determined by dissolving the isolated lactoferrin at 0.2 mg/mL. Lactoferrin samples were introduced into glass capillaries and subjected to thermal treatments at several temperatures and holding times. A sample of 20 μL was introduced per capillary, and both ends were sealed with a microflame. Capillaries were introduced by duplicate in a thermostatic bath with agitation, which maintained the temperature with a precision of ±0.1°C. After each holding time of treatment, capillaries were removed from the bath and cooled immediately in an ice water bath.

Determination of Ovine Lactoferrin Concentration

Samples after heat treatment were extracted from the capillaries, and 7 μL of every sample was introduced in each well of a 1.5% agarose gel prepared to develop radial immunodifusion as described by Sánchez et al. (1992b). The gel was prepared with 0.5% (vol/vol) of rabbit antiserum against ovine lactoferrin and the lactoferrin standards prepared at concentrations of 0.3, 0.2, 0.15, and 0.075 mg/mL.

Decimal Reduction Time and Z Values for the Denaturation of Ovine Lactoferrin

The effect of thermal treatments on the structure of lactoferrin was evaluated by determining its immunoreactive concentration along time of treatment, for each temperature and treatment medium. To calculate the kinetic parameters for denaturation of lactoferrin, the values of the logarithm of protein concentration were represented against time of treatment. The inverse of