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QUANTITATIVE DETERMINATION OF α-LACTALBUMIN AND β-LACTOGLOBULIN IN WHEY PROTEIN FRACTIONS AND OF WHEY PROTEIN CONCENTRATE IN FRANKFURTERS USING AN ELECTROIMMUNOASSAY

ABSTRACT

An electroimmunoassay was developed for quantitating whey protein concentrates incorporated into frankfurters. The simple, precise method has potential for application to a variety of other whey fortified foods. Frankfurters containing 3–7% whey protein concentrate were homogenized in 7M urea and the extract was electrophoresed on agarose plates containing antiserum to α-lactalbumin. Quantitation was obtained by measuring and comparing migration distances for the extracts and control whey concentrates; recoveries ranged from 96–105%. The concentrations of β-lactoglobulin and α-lactalbumin in milk and whey protein fractions were determined in buffer solutions using the respective antisera.

INTRODUCTION

METHODS commonly used for the quantitation of α-lactalbumin and β-lactoglobulin in products such as milk and whey are time-consuming and lack precision. Many of the fractions obtained with the acid and salt precipitation techniques represent heterogeneous mixtures. A variability of ±15–20% was obtained with immunodiffusion or electrophoretic procedures (Larson and Hageman, 1963). Electroimmunodiffusion (EID) on cellulose acetate plates was used by Sinha and Mikolajcik (1974) to establish relative differences in whey protein contents of normal and mastitic milks.

Fortification of food products with whey or whey protein concentrates (WPC) is increasing. A recent USDA proposal (Mulhern, 1976) would permit the incorporation of WPC or whey at specified levels in certain sausage products. These applications and others illustrate the need for suitable methodology for the quantitation of whey protein in a variety of fortified food products. The present paper describes a simple, precise method for quantitating α-lactalbumin (α-1a) and β-lactoglobulin (β-1g) in milk and whey, and a modification for determining the amount of WPC incorporated in heated sausage. The method, based on Laurell's (1966) electroimmunoassay (EIA) for quantitating proteins and frequently referred to as the "rocket" technique, has potential for application to a variety of whey fortified foods.

MATERIALS & METHODS

ANTISERA to α-lactalbumin and β-lactoglobulin were purchased from Antibodies Inc., California; Sea Kem Agarose was from MCI Biomedical, Marine Colloids Inc., Maine. An E-C Model 660 Immunoelectrophoresis Cell was used.

The whey protein samples were prepared by precipitating casein from skim milk at pH 4.6; the supernatant was dialyzed and whey proteins were recovered by lyophilization. A WPC prepared by ultrafiltration and Sephadex gel filtration (UFS) (McDonough et al., 1974), containing approximately 90% protein, a commercial WPC containing about 50% protein, and a heat coagulated WPC (HC-WPC) (Panzer et al., 1976) were utilized in this study. Bovine milk was obtained from a local dairy. Electrophoretically pure β-lactoglobulin and α-lactalbumin were prepared by standard procedures (Aschaffenburg and Drewry, 1957; Bengtsson et al., 1962; Groves, 1965).

Frankfurter preparation

Frankfurters were prepared essentially as described by Townsend et al. (1971) using a Schnell Kutter meat chopper and adding the commercial WPC (mixed with an equal weight of water) to an emulsion containing 3.57 kg beef, 2.08 kg pork, 2.60 kg fat, 2.25 kg ice, 1.25g sodium nitrite, 4.37g sodium ascorbate, 163g sugar, 207g salt and 43g spice. The franks were heated and smoked for about 90 min until the internal emulsion temperature reached 71°C. They were then stored at −20°C, and analyzed within 2 wk. Corrections were made for water loss during cooking in calculating the amount of WPC in the sausage.

Buffer

Barbital buffer, ionic strength 0.025, pH 8.2, was prepared by combining 7.92g sodium barbital, 1540 ml distilled water and 460 ml 0.025N hydrochloric acid (Crowle, 1973).

Electroimmunoassay

A modification of Laurell's (1966) "rocket" technique was followed. Two thoroughly cleaned 7.5 × 5 cm glass slides were coated with hot 0.2% agarose and dried at 56°C. Agarose (1% in buffer) was cooled to about 50°C prior to addition of antiserum. The slides were placed on a level surface, and 4 ml of agarose containing antiserum were applied to each slide and allowed to set for about 2 min. The slides were stored at 5°C in a humid atmosphere.

The antiserum concentration was generally 3% anti-α-lactalbumin (high titer) or 9% anti-β-lactoglobulin (medium titer). In assays of α-lactalbumin in frankfurters, 10% anti-α-lactalbumin (high titer) was used.

A series of wells (3 mm diam) were formed on each agarose slide, and antigen (4 μl) was applied to each well using a Drummond Ziptrol micro pipet with disposable capillaries. The slides were electrophoresed in barbital buffer, ionic strength 0.025, pH 8.2 at 125 volts and 5°C, generally for 60 min or for 110 min for frankfurter samples.

The precipitate loops could usually be observed by holding the slide to a light; however, if staining was desired in order to more accurately measure the peak heights (or areas), the slides were immersed for 24 hr at 5°C in 0.9% NaCl (pH 7.4–8.2) and rinsed with distilled water to wash a large proportion of unreacted antiserum proteins from the gel. The slides were then placed in 0.1% Coomassie Blue R in 25% trichloroacetic acid (Kahn and Rubin, 1975) for 3–5 min, then destained in 5% acetic acid for about 10 min. The gels were allowed to dry, and the distance from the center of the origin to the tip of the loop of precipitate was measured. Results were calculated from a graph obtained by plotting the peak heights of the standards as a function of concentration on semilogarithmic paper. Generally, standards contained 0.10–0.25 mg α-lactalbumin/ml or 0.15–0.40 mg β-lactoglobulin/ml. Of course, the concentration of the samples assayed and length of electrophoresis time are related to antibody concentration, and conditions can be established which would permit quantitation of other protein levels (Laurell, 1966).

Sample preparation

A 0.09% whey protein solution in barbital buffer was used for determination of β-lactoglobulin, while a 0.11% solution was used in the α-lactalbumin assays. The commercial WPC was analyzed at a concentration of 0.23%. A 0.4 ml aliquot of bovine milk was diluted with 2.6 ml buffer for the electroimmunoassay. HC-WPC (0.25%) was homogenized in a hand tissue grinder in 8M urea, pH 6.2 to effect solution of the protein.

The casing and outer dark layer were removed from about 100g of partially thawed frankfurters, which were then cut into pieces and ground in a Waring Blendor. A sample (about 20.00g) was immediately homogenized in 80 ml 7M urea, pH 6.2 for 2 min. The homogenate was strained through a wire mesh strainer, and a portion was passed through glass wool. A 0.3 ml aliquot was diluted with 0.9 ml barbital buffer within 10 min after initial exposure to urea. (Samples exposed to urea for 30 min before dilution gave similar results for α-lactalbumin.) The sample from the frank containing 7.3% WPC was further diluted with 1.45 ml urea-buffer (1:3). A series of WPC standards, generally 1.5–3

mg/ml in urea-buffer (1:3), were run on the same slide as the sample being examined.

Commercial WPC in water, pH 6.6 (1:2 W/V), was heated at 119°C for 1.5 min, then at 120–121°C for 5 min (in 12 × 75 mm test tubes covered with aluminum foil), mixed with barbital buffer or with 8M urea, pH 6.2, and the solutions (1.3 mg WPC/ml) were subjected to the electroimmunoassay with anti-α-lactalbumin (13%, medium titer). Samples of commercial WPC in water (1:2) were also heated as above at 121–125°C for 8 min, and assayed for α-lactalbumin.

RESULTS & DISCUSSION

DURING THE DEVELOPMENT of an approach to quantitate WPC in heated sausage, there was a distinct possibility that whey protein denaturation during the manufacture of the sausage might prevent their reaction with native whey protein antisera. Heat denatured α-lactalbumin and β-lactoglobulin did not react with antisera to native proteins in studies using immunodiffusion methods (Lyster, 1970). β-Lactoglobulin was denatured by heat more readily than α-lactalbumin. However, it was conceivable that the temperatures used in the present investigation for the preparation of frankfurters (71°C) would not sufficiently denature α-lactalbumin in the WPC to seriously interfere with its reaction with its antiserum.

Whey and milk protein

Table 1 shows the α-lactalbumin and β-lactoglobulin contents determined in whey protein fractions, WPC, and bovine milk samples (in buffer) using the electroimmunoassay. The values obtained were similar to those previously reported (Cerbulis and Farrell, 1975; Larson and Hageman, 1963; Parry, 1974; Rolleri et al., 1956). Direct comparison of values obtained in this study by electroimmunoassay could not be directly compared with those previously reported by immunodiffusion and electrophoresis procedures, since the latter only provide estimates of the amount of protein present (Larson and Hageman, 1963). In whey protein samples, α-lactalbumin ranged from 11.5–17.8% and β-lactoglobulin from 41.7–55.8%. Similarly, large within and between breed differences in the whey proteins have been reported by others (Cerbulis and Farrell, 1975). The UFS-WPC (90% protein) showed 16.4% α-1a and 56.1% β-1g. Analysis of the commercial WPC (50% protein) gave values of 7.9% α-1a and 21.0% β-1g (% of total dry matter). The coefficient of variation (C.V.) was 2%. The other whey proteins include immunoglobulins, serum albumin and lactoferrin. Pasteurization of WPC (78.2°C for 15 sec or 62.4°C for 30 min) results in approximately 20% protein denaturation, with α-lactalbumin being more heat resistant than β-lactoglobulin. Other treatments essential to production of a powder, e.g., preheating, evaporation, and spray drying, do not significantly alter protein solubility (McDonough et al., 1974). Three raw bovine milk samples contained from 0.9–1.1mg α-lactalbumin/ml, while pasteurized, homogenized milk contained 1.1 mg α-lactalbumin/ml. Raw milk samples contained 3.0 and 3.1 mg β-lactoglobulin/ml and pasteurized milk 2.5 mg/ml.

Table 1—α-Lactalbumin and β-lactoglobulin content of milk and whey protein samples

Sample	% α-1a	% β-1g
Whey protein 1[a]	12.2 ± 0.1[b]	43.2 ± 0.2
Whey protein 2	13.3 ± 0.2	42.9 ± 0.2
Whey protein 3	11.5 ± 0.2	41.7 ± 0.2
Whey protein 4	14.2 ± 0.2	55.8 ± 0.1
Whey protein 5	14.4 ± 0.1	51.5 ± 0.2
Whey protein 6	12.5 ± 0.1	50.1 ± 0.1
Whey protein 7	17.8 ± 0.1	51.6 ± 0.1
UFS[c]	16.4 ± 0.1	56.1 ± 0.2
Commercial WPC[d]	7.9 ± 0.1	21.0 ± 0.2
	α1a (mg/ml)	β-1g (mg/ml)
Cow 1 milk (raw, whole)	1.0 (1.0)[e]	3.1 (3.1)
Cow 2 milk (raw, whole)	0.9 (0.9)	ND[f]
Bulk milk (raw, skim)	1.1	3.0
Pasteurized, homogenized milk	1.1 (1.1)	2.5 (2.5)

[a] Whey protein samples 1–7 were prepared by precipitating casein from skim milk at pH 4.6, then dialyzing and lyophilizing the supernatant.
[b] Mean of duplicates and mean deviation around the median
[c] Prepared by ultrafiltration and gel filtration; contains about 90% protein
[d] Contains about 50% protein
[e] Duplicate sample
[f] Not determined

Heated samples extracted in urea

When commercial WPC was mixed with commercial frankfurters and homogenized in barbital buffer without urea (pH 8.2), quantitative results were obtained in the electroimmunoassay using antiserum to α-lactalbumin. However, when the WPC was added to the sausage emulsion and then smoked and heated, no visible immunological reaction was observed with a buffer homogenate of the sample. Possibly because of interactions with meat proteins, the antigenic sites required for precipitation were unavailable for reaction with the antibodies. The smoking and heating conditions might also have denatured the whey proteins to the extent that they would not react with the antiserum. When fortified, heated sausage was homogenized in 7M urea and the extract (diluted with buffer) subjected to the electroimmunoassay with antiserum to α-lactalbumin, quantitative results were obtained for the WPC. No differences in peak heights were found between control WPC and urea extracts of fortified frankfurters. Exposure of α-1a to urea, which promotes swelling of the protein molecule (Kronman, 1967) and disruption of weak binding forces, induces exposure of some antigen ligands and allows a precipitating lattice to form with anti-α-lactalbumin. Compared to the amount of antiserum (3%) required for measuring undenatured α-lactalbumin in milk and whey, a higher concentration (10%) was necessary with the frankfurter samples in order to detect the peaks visually. Formation of an immunoprecipitate involves a complex collective interplay of many different antibodies in varying concentrations in an antiserum to their correspondingly different ligands on a particular antigen molecule (Reimer et al., 1970). Generally, the more ligands there are on an antigen, the more readily a precipitate forms. The high concentration of urea probably causes unfolding of the partially denatured whey protein to expose only a percentage of the antigen sites; therefore, a larger concentration of antiserum must be present in order to supply sufficient corresponding precipitins to form a lattice.

Two immunoprecipitation peaks, one inside the other, were formed with the unheated commercial WPC in urea:buffer reacting with anti-α-1a. The outer peak was lighter and comprised the major portion of the "rocket" formed with the cooked sausage. The two peaks could represent an electrophoretically heterogeneous protein formed on heating. The darker, lower peak presumably contained a greater number of antigenic ligands, some of which were lost on heating the WPC. Similar patterns were reported by Kroll and Thambia (1969) for serum β_{1C}–β_{1A}-globulin. In serum, β_{1C}-globulin is converted to β_{1A}-globulin, which loses some of the antigenic sites present in the β_{1C}-globulin. In a mixture of β_{1C}–β_{1A}-globulin, the level of the highest peak gave a quantitative measure of the total amount of antigen applied.

In contrast to the results obtained with the frankfurters, when raw bovine milk was heated at 85°C for 20 min, diluted with 14 parts of 7M urea and assayed, the α-lactalbumin was denatured to such an extent that the decreased migration

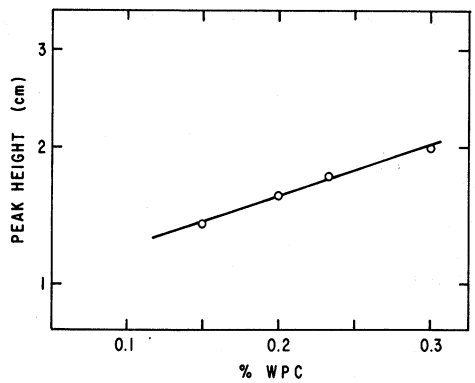

Fig. 1—Electroimmunoassay of a commercial whey protein concentrate in urea:buffer (1:3). Electrophoresis was for 110 min with 10% anti-α-lactalbumin (high titer).

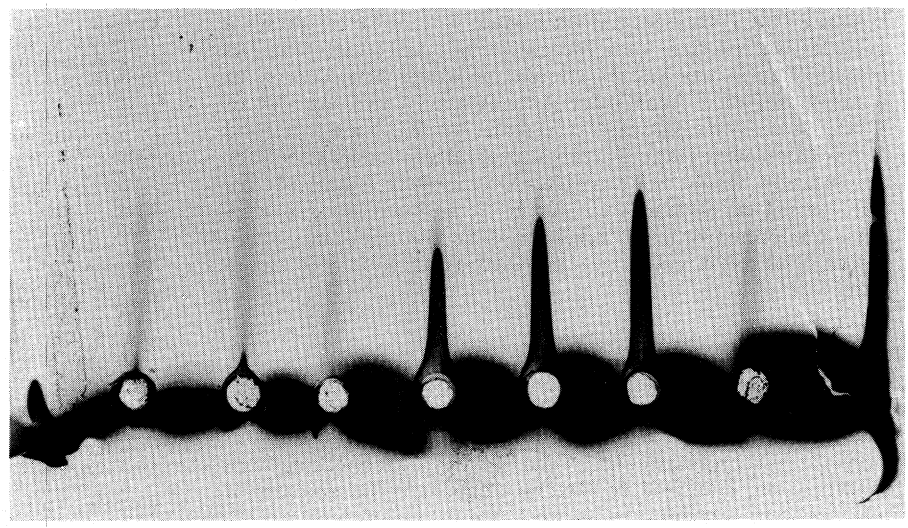

Fig. 2—Immunoelectrophoretic patterns of commercial WPC (dark peaks) and extracts of WPC-fortified frankfurters (light peaks).

distance showed that only about 25% of the protein was reacting with the anti-α-lactalbumin.

In order to study the effect of heating and smoking on the light immunoprecipitin peak observed with the fortified frankfurters, a mixture of commercial WPC, water, sodium ascorbate, and sodium nitrite (350:175:4.4:1.2) was heated at 71°C for 90 min (in frankfurter casing) or for 180 min (with and without smoking), and when homogenized in urea, the solution gave the same peak height by EIA against anti-α-lactalbumin as unheated samples of the same concentration. The portion heated for 180 min gave a predominately light immunoprecipitate as was observed with the fortified frankfurters. The 90 min sample gave an inner peak lower than that obtained with unheated WPC. Smoking produced no effect on the inner peak.

No difference in rocket lengths was observed when a mixture of commercial WPC, water and ascorbic acid (1:1:0.08) was adjusted to pH 6.0, heated at 78°C for 50 min, and compared with unheated WPC of the same concentration. Also, WPC fortified frankfurter samples were assayed with and without heating at 79 ± 0.5°C for 20 min and showed the same peak height. Therefore, it could be expected that heating sausage to a slightly higher temperature than 71°C in processing would not prevent quantitation of WPC.

Positive, but not quantitative, reactions were obtained with buffer or urea frankfurter extracts and antiserum to β-lactoglobulin. In urea, β-lactoglobulin can form aggregates involving sulfhydryl oxidation and disulfide-interchange reactions (McKenzie and Ralston, 1971). α-Lactalbumin contains no free sulfhydryl groups. It is possible that interactions of the β-lactoglobulin free –SH during sausage heating were responsible for the inability to quantitate β-lg in the frankfurters. Because of the results discussed above, α-lactalbumin, rather than β-lactoglobulin, was selected as the assaying agent.

WPC heated above 100°C

When commercial WPC in water (1:2 W/V) was heated at 119–121°C for 6.5 min, and its buffer or 8M urea extracts were assayed for α-lactalbumin, the rocket lengths obtained for the same concentration of heated and unheated WPC were identical. However, after the WPC in water (1:2) was heated at 121–125°C for 8 min and the coagulated protein was homogenized in a tissue grinder in buffer or 8M urea, less than 10% of the α-lactalbumin found in unheated samples was detected. A whey protein concentrate (about 64% protein) prepared by heating whey to 120°C (pH 5.5–6.5) for 8 min was studied using the electroimmunoassay. A series of four runs gave 3.6% (C.V. 2%) α-lactalbumin in the HC–WPC, indicating that about 5.6% of the protein had reacted with antiserum to native α-lactalbumin. Because no information on the amount of individual proteins in the WPC was available, the portion of the α-lactalbumin reacting could not be determined. The HC–WPC was prepared by recovering the heat coagulated protein by centrifugation; therefore, it is conceivable that β-lactoglobulin constituted the majority of the protein since it is more readily heat coagulated. It is apparent that heating WPC above 121°C causes denaturation of most of the α-lactalbumin and prevents reaction with native antiserum under the conditions described.

Frankfurters

Figure 1 shows the linearity of response found with the commercial WPC in urea:buffer (1:3) electrophoresed into agarose containing anti-α-lactalbumin under the conditions used for the frankfurter assays. Immunoelectrophoretic patterns obtained with the commercial WPC standard and extracts of WPC-fortified frankfurters are shown in Figure 2. Results obtained by analysis of WPC content of sausage, using antiserum to native α-lactalbumin are given in Table 2. Commercial WPC added to frankfurters at levels of 3.2–7.3% could be measured with a recovery of 96–105% and a coefficient of variation of 3%. Other levels of WPC additions could be measured using alterations in conditions of analysis. The smoked,

Table 2—Whey protein concentrate in frankfurters determined by reaction with anti α-lactalbumin

	WPC content (% of total ingredients in the finished product)	
Sample	Added	Found
Frank 1	3.24	3.12 ± 0.08[a]
Frank 2	3.60	3.52 ± 0.10
Frank 3	3.80	4.00 ± 0.11
Frank 4	4.40	4.38 ± 0.12
Frank 5	7.30	7.50 ± 0.10

[a] Mean of duplicates and mean deviation around the median

heated (71°C) sausage was extracted with urea to obtain a quantitative reaction with the antiserum. Since different lots of WPC could vary in α-1a content, it would be necessary to compare fortified frankfurter samples against the same lot of WPC used in the sausage. If samples of WPC used in food fortification were retained, the appropriate standards need for quantitation would be available. Alternatively, standards for WPC's used in frankfurters could be established, e.g., specifying a predefined ratio of lactose to α-lactalbumin, and fortification with WPC could be expressed as amount of α-lactalbumin.

SUMMARY

A SIMPLE, precise electroimmunoassay was developed for the quantitation of the principal whey proteins using antisera to β-lactoglobulin and α-lactalbumin. In addition to the quantitation of these proteins in milks and in wheys, the method, with modification, could be utilized to quantitate the amount of whey protein concentrates incorporated into frankfurters.

For the quantitation of WPC in frankfurters, α-lactalbumin antiserum was used because of the increased resistance of α-lactalbumin to thermal denaturation compared with β-lactoglobulin. Identical results were obtained for α-lactalbumin in WPC heated at 119–121°C for 6.5 min as that found in unheated controls. However, after heating at temperatures above 121°C, less than 10% of the original α-lactalbumin was detected. Since WPC's vary in their α-lactalbumin content, it was necessary to use the same concentrate for the standard as was used in frankfurter fortification. After heating the frankfurter-WPC emulsion to 71°C, it was necessary to extract the frankfurter with 7M urea to reduce the protein interactions and free the antigen ligands. A relatively high concentration of anti-α-lactalbumin was necessary to form visible precipitation loops using the frankfurter extract. The method was used successfully to quantitate WPC (96–105% recoveries) in frankfurters containing 3–7% WPC. With appropriate modification, the method should be applicable for the quantitation of other whey-fortified products.