PHYSIOLOGY AND MANAGEMENT

Enzyme-Linked Immunosorbent Assays for Bovine α-Lactalbumin and β-Lactoglobulin in Serum and Tissue Culture Media

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ABSTRACT

Enzyme-linked immunosorbent assays for bovine α-lactalbumin and β-lactoglobulin have been developed for measurements of serum and tissue culture samples. Either α-lactalbumin or β-lactoglobulin antiserum was coated on ELISA plates. Biotinylated proteins were used in competition with unknown amount of proteins in samples. After unbound proteins were washed off, ExtrAvidin-peroxidase and tetramethylbenzidine were then used as a detection system. Crossreactivity of caseins or bovine serum albumin was less than 0.0001% in either α-lactalbumin or β-lactoglobulin ELISA. Parallel curves from serial dilutions were obtained in serum and media samples. The additivity of α-lactalbumin and β-lactoglobulin ELISA was validated in either serum or medium samples. The intraassay and interassay coefficients of variation for α-lactalbumin and β-lactoglobulin ELISA were below 10% over 51 and 47 assays. The ELISA are useful in mammary gland biology studies for measuring milk whey protein in serum or culture media. (Key words: enzyme-linked immunosorbent assay, α-lactalbumin, β-lactoglobulin)

INTRODUCTION

Owing to its role in lactose synthetase activity (5), α-lactalbumin has been used as an index of mammary gland function in response either to hormonal regulation in bovine explant culture (1, 8) or as an index of udder development and function (12). A simple and sensitive assay to measure either α-lactalbumin or β-lactoglobulin in either serum or explant culture in the studies of hormonal regulation of mammary function and development would have great utility. Enzyme-linked immunosorbent assay provides the advantages of radioimmunoassay (RIA), and, in addition, it is possible to determine α-lactalbumin or β-lactoglobulin concentrations semiquantitatively under field conditions without sophisticated or expensive equipment.

Developments in enzyme immunoassay (EIA) and ELISA procedures have resulted in enzymatic alternatives to RIA for the measurement of several hormones and soluble proteins (13, 18). High concentrations of bovine α-lactalbumin and β-lactoglobulin in milk and mammary secretions can be measured by densitometric scanning after PAGE (14) or by immunodiffusion assay (9). A RIA for measurement of bovine α-lactalbumin in serum, milk, and tissue culture media has been reported by Akers et al (2). The RIA and a noncompetitive EIA of human α-lactalbumin have also been studied (6, 17, 19). An indirect ELISA for detection of bovine milk protein in ovine milk and cheese has also been described (7, 16). Using a different strategy, the competitive ELISA are introduced in this study for the measurement of α-lactalbumin and β-lactoglobulin in serum and tissue culture samples.

Abbreviation key: DAB = diaminobenzidine, EIA = enzyme immunoassay, RIA = radioimmunoassay, TMB = tetramethylbenzidine.

MATERIALS AND METHODS

ELISA

The outline of competitive ELISA procedure is described in Figure 1. Nunc-Immuno Plate IF (MaxiSorp, with certificate) was purchased from VWR Scientific (Chicago, IL). Acetate plate sealer was purchased from Flow
ASSAYS FOR MILK PROTEIN

Laboratories (McLean, VA). Rabbit anti-bovine α-lactalbumin antiserum was obtained from Isabel A. Forsyth (Reading, Engl.). Rabbit anti-bovine β-lactoglobulin antiserum was purchased from Nordic Immunological Laboratories (Capistrano Beach, CA). Milk proteins, enzymes, and other ELISA reagents were purchased from Sigma (St. Louis, MO) except when specified otherwise.

Stock Solutions

Stock solutions are described as follows.

Coating buffer: .05 M sodium carbonate-bicarbonate, pH 9.6. Assay buffer: .04 M 3-[N-morpholino]propanesulfonic acid, .12 M sodium chloride, .01 M EDTA, .1% (wt/vol) gelatin (porcine skin), .05% (vol/vol) Tween 20, .005% (vol/vol) chlorohexidine digluconate, .5 mg/L Leupeptin (Boehringer Mannheim, Indianapolis, IN), pH 7.4. Wash buffer: .12 M sodium chloride, .02 M sodium phosphate, .025% (vol/vol) Tween 20, pH 7.4. Substrate buffer: .2 ml of 3,3',5,5'-tetramethylbenzidine (TMB) stock solution (20 mg of TMB/ml of dimethyl sulfoxide), .064 ml of .5 M hydrogen peroxide, 19.74 ml of .05 M sodium acetate, pH 4.8 (prepare immediately before use).

Biotin Conjugate Preparation

Purified α-lactalbumin (7.1 mg) or β-lactoglobulin (9.1 mg, containing of 50% A and 50% B form) were dissolved in 1 ml of 50 mM sodium bicarbonate buffer (pH 8.3). Sulfocon junimidy 6-biotinamidohexanoate (1.39 mg, NHS-LC-Biotin, Pierce, Rockford, IL) was added and incubated at 4°C for 3 h. The reactions were stopped by adding 20 μmol of ammonium bicarbonate, and the free biotin or unreacted compounds were separated from conjugates by Centricon-10 (Amicon, Danvers, MA) and G-25 columns.

Protocol of α-Lactalbumin and β-Lactoglobulin ELISA

The protocol for ELISA of α-lactalbumin and β-lactoglobulin follows:

1. Coat 100 μl of bovine α-lactalbumin antiserum (1:50,000 dilution in coating buffer) or bovine β-lactoglobulin antiserum (1:10,000 dilution in coating buffer) on Nunc-Immuno plates.
2. Cover with acetate plate sealer and incubate overnight at 4°C.
3. Wash four times with wash buffer.
4. For immediate use, add 50 μl of assay buffer into each well. For later use and storage, add 200 μl of assay buffer into each well. Store at 4°C in a humidified chamber. Go back to step 3 in later assay.
5. Pipette 50 μl of α-lactalbumin or β-lactoglobulin standards or diluted samples (in assay buffer) in triplicate into nonadjacent wells.
6. Add either 50 μl of 1:100,000 diluted (in assay buffer) biotin-LC-α-lactalbumin or biotin-LC-β-lactoglobulin conjugates into each well.
7. Incubate overnight at 4°C or 4 h at 20°C.
8. Wash four times with wash buffer.
9. Add 100 μl of 1:10,000 diluted ExtrAvidin-peroxidase (in assay buffer) into each well.
10. Incubate at 20°C (or room temperature) for 2 h.
11. Wash four times with wash buffer.
12. Add 125 μl of fresh substrate into each well and incubate for 12 min for α-lactalbumin ELISA and 20 min for β-lactoglobulin at 20°C.
13. Add 50 μl of .5 M sulfuric acid to stop the peroxidase reaction.
14. Read absorbances at 450 nm minus 600 nm by EIA autoreader (Bio-Tek model EL310, Burlington, VT).
15. Receive data and analyze by four-parameter logistic fit (15) on a Macintosh computer.
General Considerations

The ELISA plates used exhibit high antibody binding and uniformity in comparison with several other brands tested. The ExtrAvidin (Sigma) has high affinity to biotin, like avidin, and low nonspecific binding, like streptavidin. The present study used TMB as substrate because it is a sensitive, safe, and convenient means of detecting peroxidase activity (4). The TMB is an nonmutagenic chromogen for horseradish peroxidase in EIA (4), whereas several other peroxidase substrates, including 2,2'-azino-bis(3-ethylbenzthiazoline-sulfonic acid) and o-phenylene diamine have been shown to be mutagenic (3, 20). The TMB substrate solution tends to precipitate upon storage, and we have found it essential to use disposable reservoirs and to make fresh substrate solution within 2 min of use.

Immunoblotting

Milk protein standards were separated by SDS-PAGE (14%, reducing gel) and transferred onto Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked by ELISA assay buffer for 30 min. Bovine α-lactalbumin or bovine β-lactoglobulin antiserum (1:10,000 diluted in assay buffer) was added and incubated at 20°C for 2 h. Membranes were washed and goat anti-rabbit IgG-peroxidase (1:5,000, Sigma) added and incubated for another 2 h at 20°C. Membranes were washed again, and diaminobenzidine (DAB) substrate solution (12.5 mg of DAB, .5 ml of 1% CoCl₂, 25 µl of 30% H₂O₂, 24.5 ml of phosphate-buffered saline, pH 7.4) were added. After a distinctive signal and background were formed, the color reaction was stopped by submerging the membrane into 1 mM EDTA solution.

RESULTS AND DISCUSSION

The crossreactivity of rabbit anti-bovine α-lactalbumin and β-lactoglobulin antiserum to other milk proteins is shown in Figure 2 by immunoblots. There was a crossreactivity between α-lactalbumin antiserum and β-lactoglobulin of about 10%. Neither antisera crossreacted with either bovine serum albumin or caseins at 1% level tested by immunoblots. The crossreactivity of major milk proteins in α-lactalbumin and β-lactoglobulin ELISA is

![Figure 2. Coomassie staining of milk protein serial dilutions and their immunoblots tested by α-lactalbumin or β-lactoglobulin antiserum. Part a: Coomassie staining of milk proteins. Lane 1: bovine serum albumin (BSA), α-casein (CN), β-CN, x-CN, β-lactoglobulin (β-LG), and α-lactalbumin (α-LA). 2 µg of each; lane 2: 1 µg; lane 3: .5 µg; lane 4: .25 µg; lane 5: .125 µg; lane 6: .063 µg; lane 7: .031 µg; lane 8: .016 µg. Part b: α-lactalbumin immunoblot on serial diluted milk proteins. Part c: β-Lactoglobulin immunoblot on serial diluted milk proteins.](image)
Table 1. Crossreactivity of α-lactalbumin (α-LA), β-lactoglobulin (β-LG); α-casein (CN), β-CN, and κ-CN; and bovine serum albumin (BSA) to α-LA and β-LG ELISA and the detectability (or sensitivity) for these systems.

<table>
<thead>
<tr>
<th>Crossreactivity</th>
<th>Detectability (pg)</th>
<th>α-LA</th>
<th>β-LG</th>
<th>α-CN</th>
<th>β-CN</th>
<th>κ-CN</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-LA ELISA</td>
<td>25</td>
<td>.0003</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>β-LG ELISA</td>
<td>100</td>
<td>&lt;.0001</td>
<td>.0003</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Although there was some crossreactivity with β-lactoglobulin found in α-lactalbumin antisemum, the crossreactivity in competitive ELISA was only .0003%. The crossreactivity of caseins and bovine serum albumin was less than .0001% in either α-lactalbumin or β-lactoglobulin ELISA. The detectability (or sensitivity) was 25 pg for α-lactalbumin and 100 pg for β-lactoglobulin (i.e., .5 and 2 ng/ml when 50 μl of sample were added).

Parallel dilution curves from α-lactalbumin or β-lactoglobulin stock standards, medium, or serum samples are shown in Figure 3. There were no significant differences found in either medium or serum sample dilution curves when compared with standard curves of α-lactalbumin or β-lactoglobulin ELISA. Accuracy was also confirmed by adding a known quantity of α-lactalbumin or β-lactoglobulin to both medium and serum samples (Table 2). Internal controls were included in all assays to determine the intraassay and interassay CV for both ELISA. The average intraassay CV from 51 α-lactalbumin ELISA was 5.8 and 9.2% for interassay CV. The average intraassay CV from 47 β-lactoglobulin ELISA was 6.2 and 8.8% for interassay CV.

The ELISA are suitable for determining small amounts of proteins in unpurified samples, such as explant culture media (10) and serum samples (11). With appropriate adjustments, the ELISA can also be used to measure α-lactalbumin and β-lactoglobulin in samples of high concentration, such as milk or dry period mammary secretions. Concentrations of α-lactalbumin in skim milk determined by ELISA from three different cows at 8 wk postpartum were 1.14, 1.12, and 1.24 mg/ml, respectively. Concentrations of β-lactoglobulin in skim milk determined by ELISA from three different cows at 8 wk postpartum were 3.41, and 1.67 mg/ml, respectively. The values of concentrations in milk were further confirmed by Coomassie staining of electrophoresis gels and analyzed by an image analysis program (Collage™, Fotodyne, New Berlin, WI).

Serum concentrations of α-lactalbumin measured by ELISA (11) are similar to the RIA results described by McFadden et al (12).
TABLE 2. Recovery of α-lactalbumin or β-lactoglobulin added to 1/10 diluted serum or medium samples.

<table>
<thead>
<tr>
<th>Protein added</th>
<th>Serum</th>
<th>(%)^1</th>
<th>Medium</th>
<th>(%)^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ng)</td>
<td>(ng)</td>
<td></td>
<td>(ng)</td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+0</td>
<td>.017</td>
<td></td>
<td>.038</td>
<td></td>
</tr>
<tr>
<td>+.1</td>
<td>.111</td>
<td>94</td>
<td>.127</td>
<td>89</td>
</tr>
<tr>
<td>+.4</td>
<td>.462</td>
<td>111</td>
<td>.368</td>
<td>82</td>
</tr>
<tr>
<td>+1.0</td>
<td>1.023</td>
<td>101</td>
<td>1.086</td>
<td>105</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+0</td>
<td>.024</td>
<td></td>
<td>.037</td>
<td></td>
</tr>
<tr>
<td>+.1</td>
<td>.115</td>
<td>92</td>
<td>.152</td>
<td>115</td>
</tr>
<tr>
<td>+.4</td>
<td>.328</td>
<td>76</td>
<td>.422</td>
<td>96</td>
</tr>
<tr>
<td>+1.0</td>
<td>.911</td>
<td>89</td>
<td>.804</td>
<td>77</td>
</tr>
</tbody>
</table>

^1Percentage recovery.

It is possible that auto-antibodies to α-lactalbumin or β-lactoglobulin are present in bovine serum. However, the dilution parallelisms and recovery percentages from different samples would suggest that the interference of auto-antibody is small, if any, in these samples.

Because of the similarity of α-lactalbumin from different species, a commercially available polyclonal rabbit anti-human α-lactalbumin antiserum (Sigma) can be used in substitution of anti-bovine α-lactalbumin antiserum. A higher concentration is needed for anti-human α-lactalbumin antiserum (1:2000 coating on ELISA plates compared with anti-bovine α-lactalbumin antiserum (1:50,000) in order to determine bovine α-lactalbumin.

CONCLUSIONS

A competitive ELISA system in substitution for RIA has been introduced. The α-lactalbumin and β-lactoglobulin ELISA are suitable in measurement of culture and serum samples.

ACKNOWLEDGMENT

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