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Enzyme-Linked Immuno-Sorbent Assay for Bovine Caseins

Heng-Cherl Yom
Department of Dairy Science, University of Wisconsin

우 Casein의 면역효소분석법

염 행 철
워스컨신대학교 낙농학과

요 약

본 연구는 우 casein을 신속, 정확하게 분석할 수 있는 특수 면역효소분석법을 개발하였다. Biotin이 연결된 casein과 peroxidase-conjugated avidin을 사용하였으며 면역화시킨 닭의 난황으로부터 추출한 항체를 이용하여 분석하였다. Sulfo-N-hydroxy succinimido biotin을 사용하여 casein에 biotin을 연결시키고 microplate에 고정한 뒤 peroxidase-conjugated avidin을 결합시켰다. 생산된 항체는 α-와 β-casein에 특이적이었으며, 유청단백질, IgG, 우혈청 알부민과의 교차반응은 면역효소분석법과 Western blot에서 나타나지 않았다. 본 분석법의 민감도는 2ng에서 20μg이었으며 Standard와 시료의 분석시 뚜렷한 평행곡선이 형성되었다. Intra-assay와 Inter-assay의 변이계수는 각각 5.5와 5.7%이었다. 그리고 비유 초기의 casein량을 조사한 결과 분만전 3일경부터 급격히 상승하는 것을 알 수 있었다.

I. INTRODUCTION

Casein content of milk is a critical variable in most of equations for prediction of cheese yield. The proportion of casein to total protein in milk is influenced by many factors such as breed (Blake, 1980; Cerbullis, 1975), stage of lactation (Larson, 1956; Waite, 1956), season (Harland, 1955; Szijarto, 1973), bacterial proteolysis during prolonged storage (Adams, 1976; Aylward, 1980; DeBeukelar, 1977), and disease including mastitis (Ashworth, 1965; Haenlein, 1973). Caseins were originally defined as a group of phospho-proteins from raw skim milk which were precipitated at pH 4.6 and 20℃ (Jenness, 1956). The nomenclature committee (Eigel, 1984) recommends that caseins be identified according to homology of their primary structures (amino acid sequences) into four families : α_{S1}-, α_{S2}-, β-, and κ-casein.

Ashworth (1965) determined casein in milk by a dye binding method. Kindstedt et al. (1983) employed the micro-Kjeldahl method to estimate casein with nitrogen content tn milk by subtracting non-casein nitrogen from total nitrogen in milk. The conclusion was drawn that the problem of determining the casein content of milk would not be resolved completely until a rapid direct method for measuring casein is developed. Ng Kwai Hang et al. (1984) developed a rapid polyacrylamide gel electrophoretic method in the presence of urea to separate caseins in the gel. Basch et al. (1985) studied the use of SDS-PAGE to quantify the ratio of casein to whey protein, and estimated milk protein by scanning the gel by means of a densitometer. Nonetheless, a widely used method for many years is that of Rowland (1938) in which the total nitrogen of milk and non-casein nitro-

gen in a filtrate obtained after precipitating casein at pH 4.6 were determined by Kjeldahl analysis. The method is very time consuming for a large number of samples.

Recent developments in enzyme immunoassay (EIA) and ELISA have resulted in sensitive competitive immunoassay procedures for many proteins (Spearow, 1987). Development of an ELISA for a protein allows researchers more desirable alternative to radioimmunoassay (RIA) because ELISA is not associated with disadvantages such as limited shelf life of isotope, licensing, safety, and waste disposal problems. However, to date, no enzyme immunoassay for bovine casein has been reported. The protein has been difficult to assay due to its micelle structure and chemical characteristics in solution (Schmidt, 1976). Therefore, the objectives of this study were to develop a rapid and sensitive casein ELISA suitable for direct estimation of casein in milk or for physiological estimation of casein synthesized *in vitro* in either cell or tissue culture. A competitive ELISA for angiotensin-1-converting enzyme was described by Lanzillo and Fanburg (1982) and the similar principle was applied in casein ELISA developed in this study. Avidin-biotin complex(ABC) system was used in the ELISA in that biotin renders minimal effects on the biological activity of a protein, moreover, the extra-high affinity between avidin and biotin assures researchers of a readily formed stable complex between the avidin conjugate and the biotinylated protein (Fuccillo, 1985). In addition the ABC system can improve sensitivity of an ELISA due to the potential for amplification coming from the multiple binding sites. Chicken antibody was used because it was often difficult to obtain antibodies with good quality in a large quantity, in consequence, there had been demand for a better alternative method. Chicken antibody from egg yolk meets this criteria as hens lay eggs almost every day and their collection is much easier than bleeding animals. In addition, the antibody separation steps are simple and the yield is so high that one may obtain sufficient amount of specific antibody (Polson, 1980b). Consequently, in this study the feasibility of application of a chicken antibody from egg yolk in immunoassay was investigated and the ELISA developed was validated for specificity, parallelism, sensitivity, and assay variation. The ELISA was applied to estimate casein contents of periparturient bovine milk samples.

Ⅱ. MATERIALS AND METHODS

1. Materials and Reagents

EIA microplates (flat-bottomed, 96-well, polystyrene) and their plastic sealers were from Becton Dickinson and Co. (Oxnard, CA). Nunc immunowashing device was purchased from Vanguard Intenational (Neptune, NJ). Protein assay reagents for bicinconinic acid (BCA) method and sulfo-N-hydroxysuccinimido biotin (sulfo-NHS-biotin) were from Pierce Chemical Co. (Rockford, IL). Bovine casein used for immunization and biotinylation was from Sigma Chemical Co. (St. Louis, MO) and its purity was examined by polyacrylamide gel electrophoresis to show only α and β bands. α-casein, β casein, α-lactalbumin, and β-lactoglobulin from bovine milk were provided by Dr. Norman Olson (University of Wisconsin, Madison, WI). Bovine IgG and serum albumin were from Sigma Chemical Co. Freund's complete adjuvant was from Gibco Laboratories (Grand Island, NY). Polyethylene glycol (PEG) and avidin-peroxidase conjugate were from Sigma Chemical Co.

EIA autoreader was model EL-310 of Bio-Tek Instruments (Burlington, VT).

2. Micro BCA Assay

Protein assay was performed using BCA reagents and the assay was modified to reduce sample size from 100 μl to 15 μl. Standard solution was bovine serum albumin (Fraction V) from Pierce Co. Samples were diluted in double distilled H_2O and triplicated standards or samples were added to wells of an EIA microplate. Reagent A (BCA detection reagent) and reagent B ($CuSO_4$) were mixed at a ratio of 50:1. Immediately, 300 μl of the reaction mixture was added to each well. The plate was incubated at 37 ℃ in an incubator for 30 min and read at 600 nm by EIA autoreader. It was able to estimate a protein concentration of 10 μg/ml by this method. The three estimated values were averaged and their coefficients of variation of intra-assay and inter-assay were 1.6 and 5.3 %, respectively.

3. Immunization

Five white Leghorn chickens, 20 week-old single-comb, with a consistent egg laying cycle were inoculated intramuscularly in four sites with emulsified casein solution as a method described by Polson et al. (1986b). One mg of casein (α 70%, β 30%) was dissolved in 1 ml of .01 M sodium hydroxide and neutralized with .02 M sodium phosphate (monobasic) and the solution was emulsified with an equal volume of Freund's complete adjuvant. Hens were given a booster injection twice, one and three weeks after the first inoculation, with 20% volume of the first inoculation in a same manner. Preimmune eggs were collected and used for control. The eggs from the immunized hens were collected for 2 months.

4. Antibody Separation

Yolk immunoglobulin (IgY) was separated by addition of PEG (M.W. 8,000) as described by Polson et al.(1980a). Briefly, eggs were broken on a yolk separator and the egg yolks were separated from the albumin (the egg white) and thoroughly washed with .02 M phosphate buffered saline (PBS, pH 7.5). The yolk volume was measured. A volume of PBS equivalent to two times of the yolk volume was added and mixed well. The pulverized PEG was added to give a final concentration of 3.5% (wt/vol) of the diluted yolk. The mixture was stirred until PEG was dissolved, and centrifuged at 7,000 g for 20 min at 4 ℃ to give 3 layers. A clear supernatant layer with a fatty layer was decanted into a funnel with a plug of cotton. The volume of the clear filtrate was measured, more PEG was added to give a final concentration of 12% (wt/vol) of the filtrate, stirred thoroughly, and centrifuged as above. The pellet was dissolved in PBS equivalent to the original yolk volume. This IgY solution was precipitated with 12% PEG (wt/vol) and centrifuged as above. The pellet was again dissolved in PBS equivalent to the original yolk volume. The absolute ethanol was added to give 40% (vol/vol) of the solution and the mixture was centrifuged at 7,000 g 20 min at −10 ℃. The final pellet was redissolved in a volume of PBS equivalent to a half of the original yolk volume. Protein assay was performed as described above and the protein concentrations of IgY solutions ranged from 6 to 16 mg/ml.

5. ELISA Buffers

The following buffer system for casein ELISA were found to be the least variable and allowed the lowest non-specific background.

1) Coating buffer : 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6
2) Washing buffer : 0.02 M sodium phosphate, 0.05% Tween 20(vol/vol), pH 8.0
3) Assay buffer : 0.02M sodium phosphate, 0.01 M EDTA(trisodium salt), 0.1% gelatin (wt/vol), 0.05% Tween 20(vol/vol), pH 8.7
4) Tetramethylbenzidine(TMB) substrate buffer : 14.8 ml of 0.05M potassium acetate, pH 5.0; 50 μl of 0.5 M hydrogen peroxide, and 150 μl of TMB 10 mg/ml of dimethylsulfoxide
5) Stop buffer : 0.5 M Sulfuric acid

6. Biotinylation of Casein

Casein(α 70%, β 30%) was dissolved as described above and neutralized with 0.02 M sodium phosphate(monobasic) to pH 7.3. The solution was biotinylated with sulfo-NHS-biotin. To optimize the biotinylation of casein, several molar ratios of the biotin to casein were evaluated. The biotin in the molar ratio of 3 to 300(sulfo-NHS-biotin to casein) was added to test tubes containing 1 mg casein in solution. The reaction mixture was mixed well until the biotin was dissolved, and incubated for 1 hr at room temperature(20 ℃). A 500 μl aliquot of the reaction mixture was applied to a coarse G-25 Sephadex column(1.0 × 20 cm) to separate the free biotin from the bound. Twenty fractions of 0.5 ml, excluding the void volume of blue dextran, were collected by a fraction collected by micro BCA assay. The three fractions that comprised the center of the protein peak were pooled and diluted with the coating buffer to give a final concentration of 3 μl/ml. A 100 μl aliquot of diluted fractions was added to an EIA microplate and the plate was incubated for 2 hr at room temperature. Unbound biotinylated casein was removed by washing four times with the washing buffer with the Nunc immunowashing device with a vacuum pump. Immediately, 125 μl of avidin-peroxidase conjugate(250 ng/ml) diluted in the assay buffer was added. The plate was shaken gently, incubated for 2 hr at room temperature, and washed with the washing buffer 8 times. A 125 μl aliquot of TMG substrate was added to each well and incubated for 30 min. The microtiter plate was read at a wavelength of 600 nm by the EIA autoreader.

7. Antibody Titration

The titer of the separated IgY was tested by ELISA developed in this study. Several concentrations of immune IgY or preimmune IgY were diluted in the coating buffer to optimize signal and background. The wells with preimmune IgY were used as blanks and the background was neglible at 3 μg/ml of IgY. A 100 μl aliquot of diluted IgY was added to each well of an EIA microplate and incubated for 2 h at room temperature. Unbound IgY was removed by washing four times with the washing buffer. Biotinylated casein was diluted with the assay buffer to give a final concentration of 100 ng/ml. Its 125 μl aliquot was added to each well immediately after washing and the plate was shaken gently and incubated for 2 hr at room temperature or overnight at 4 ℃. A 125 μl of avidin-peroxidase conjugate(500 ng/ml) diluted in the assay buffer was added. The remaining steps were the same as described above in the biotinylation section. However, the reaction was stopped by adding 50 μl of the stop buffer after 30 min and the plate was read at dual wavelengths of 450~600 nm by the EIA autoreader.

8. Casein Standard Preparation

It was not suitable to use lyophilized casein as a reference standard due to its instability over storage in 4℃ or −20℃. The solubility of caseins depends on many factors such as pH, ionic strength, Ca ions, temperature, κ-casein concentration, etc. (Schmidt, 1976). Various buffers such as high pH(>8.0), low ionic strength(<0.5M), and calcium free were used to attempt to maximize conditions for stability and solubility of the lyophilized casein.

To overcome the problem of solubility, a pool of milk samples was used as a standard. Milk samples from 15 Holstein cows with low somatic cell count were collected and pooled. A 0.05 mol EDTA(trisodium salt) was added to chelate divalent ions and thus to disaggregate the casein micelles. The solution was vigorously stirred for 10 min and centrifuged at 50,000 × g for 20 min at 4℃ to separate fat globules. The protein concentration of skim milk was determined by the micro BCA assay. The reference standard was stable for at least 2 weeks. The calculation of a reference standard of milk was based on the report from Barbano(1985). Bovine milk protein consists of 82% casein and 18% whey protein. Casein consists of α 48%, β 36% and κ 15%. Thus, the following formula was used to calculate the percentage of α and β casein :

$$(\alpha+\beta)\ \text{casein}\ \% = (\text{skim milk protein}\ \%) \times 0.82 \times 0.84$$

where 0.82 is casein fraction and 0.84 is α and β fraction.

The series of standards were prepared by diluting the milk standard with the assay buffer.

9. Casein ELISA

A 100 μl aliquot of chicken IgY against casein and preimmune IgY as blank, diluted in coating buffer(3 μg protein/ml), were added to each well of an EIA microtiter plate. The plate was sealed with a plate sealer, incubated for 1 d at 4℃, and washed with washing buffer four times. At this stage the IgY-coated plates can be stored in 125 μl/well of 0.02 M sodium phosphate buffer, pH 8.0, for a month at 4℃. A 65 μl biotinylated casein diluted in the assay buffer(1 ng/ml for the most sensitive assay) was added to the wells. The concentration of biotinylated casein may vary depending on sensitivity(see the result and discussion). Immediately, 65 μl of a serial standard or sample was added to each well with an effort to standardize a time interval. The plate was shaken gently, sealed with a plate sealer, incubated either overnight at 4℃ or for 2 hr at room temperature and washed 4 times. The addition of avidin-peroxidase conjugate and the remaining steps were the same as described in the antibody titration section.

10. Estimation of Casein in Peripaturient Milk

Milk samples were taken from 2 pregnant Holstein cows which were in their dry period after a second lactation. The samples were collected from 4 quarters and pooled once a day for about 3 weeks before parturition and twice a day for about 2 weeks after parturition. The addition of EDTA and centrifugation were done as described above in the casein standard preparation section. The micro BCA was performed to estimate total protein concentration and ELISA was carried out to estimate casein proportion. The biotinylated casein concentration added was 500 ng/ml. Three independent assays were perforrmed with triplication each time and their values were averaged.

Ⅲ. RESULTS AND DISCUSSIONS

1. Titer of Anti-Casein IgY

Three hens out of the five immunized showed satisfactory immune responses to caseins, while one responded insignificantly and the other stopped laying after the second inoculation. The titer of IgY against casein expressed as absorbance vs. time in weeks after immunization is shown in Fig. 1. The titer of IgY after the first and the second booster injection increased in a stepwise manner reaching a peak between 4 to 6 weeks after the immunization. The second booster injection was more effective in raising the titer than the first one. The IgY titer reached a peak between 1 to 3 weeks after the second booster injection indicating a delayed response. This pattern is peculiar in that the immune response in egg yolk to the booster injection was much slower than that of domestic animals in serum. This could be due to a delay of antibody accumulation in the ovary from the peripheral circulation.

2. Biotinylation

The optimal molar ratio of sulfo-NHS-biotin to casein was between 3 to 30 : 1 as shown in Fig. 2. In casein ELISA the biotinylation was performed at a ratio of 30 : 1. However, the ratio of 60 or more : 1 showed the substantially reduced absorbance. This may be explained due to the fact that the higher molar ratio contains the more biotin molecules binding per casein,

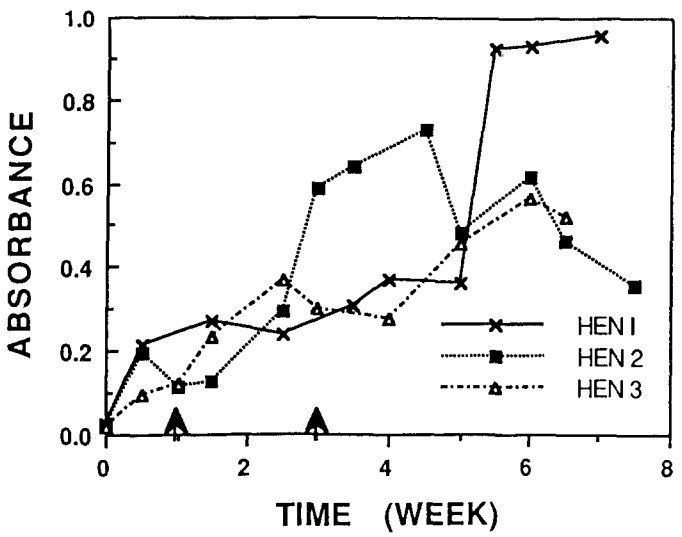

Fig. 1. The titer of anti-casein IgY from egg yolks of immunized hens. The titer estimated by ELISA is expressed as absorbance at dual wavelengths of 450~600 nm vs time in week after immunization. A 100 μl of μg/3ml IgY was coated on each well in the microplate. The concentrations of biltinylated casein and avidin-peroxidase were 100 ng/ml and 500 ng/ml, respectively. The arrows indicate the times at which the the booster injections were given. Each point represents overall mean of 2 separate experiment results with 5 replications each.

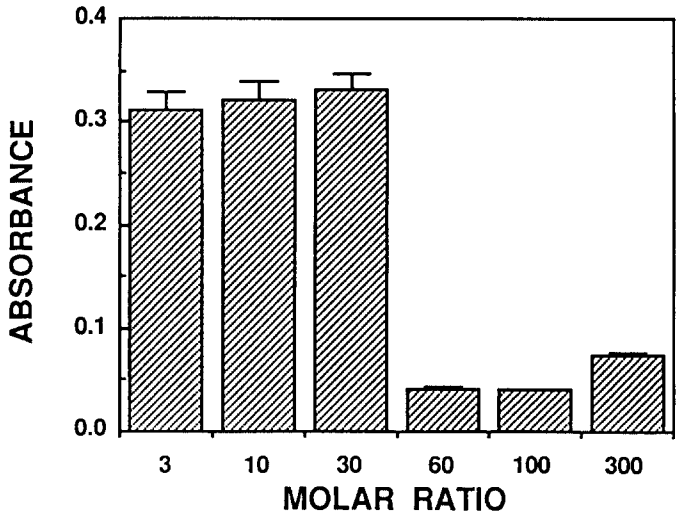

Fig. 2. The effect of biotin to casein molar ratios on absorbance at 600nm. Each bar represent overall mean of 3 independent experiment results with 5 replications each.

resulting in a conformational change, thus, interfering in the reaction between the antigen and antibody due to a steric hindrance.

3. Validation of ELISA

1) Specificity

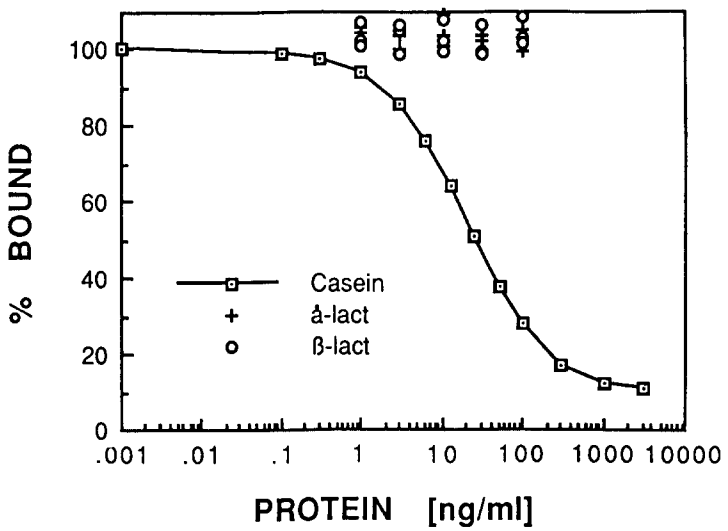

Fig. 3. The specificity of chicken anti-casein IgY expressed as % bound vs. protein concentration. The antibodies appeared to be bound only to caseins thus allowing casein-specfic displacement curve and their cross-reactions with α-lactalbumin(α-lact) and β-lactoglobulin (β-lact) at concentrations of 1 to 100ng/ml were undetectable. The points represent the means of 5 replications.

The specificity of antibody used in casein ELISA was examined to ensure that only casein was measured in the assay. The whey proteins in milk such as α-lactalbumin and β-lactoglobulin at concentrations of 1 to 100 ng/ml were incubated with 1 ng/ml of biotinylated casein and the diluted IgY coated on an EIA microplate in order to test that neither of these proteins displaced the biotinylated casein for IgY against casein. As the specificity is shown in Fig. 3, none of the above whey proteins showed detectable cross-reactivity with the IgY in the casein ELISA. The antibodies were analyzed for cross-reaction by Western blot (data not shown) and they were only specific for α- and β-casein. They did not cross-react with κ-casein, α-lactalbumin, β-lactoglobulin, bovine serum albumin, and bovine IgG.

2) Parallelism

Casein standard(see Casein Standard preparation) and either milk samples or sera both from Holstein cows were serially diluted with the assay buffer and performed the casein ELISA to verify that a parallelism existed between the curves of casein standard and samples. The sera were obtained from cows 10 d prepatum because they may have absorbed casein via their mammary glands. Casein ELISA for the parallelism studies was performed on the same microplates and under the same reaction conditions. Fig. 4. A shows a parallelism between the standard and milk samples. The slopes and their standard errors of the curves for standard and milk samples were 1.24 ± 0.30 and 1.00 ± 0.22, respectively. The regression coefficients for the linear portions of the two curves were not different ($p<0.05$). Fig. 4.B shows also a parallelism between the standard and serum samples. The sera were diluted in the assay buffer 1/1,000,000; 1/100,000; 1/10,000; 1/1,000; 1/100, 1/10, and 1/3, respectively, and plotted along with standard. The slopes and their standard errors of the two curves for standard and serum samples were 0.64 ± 0.24 and 0.74 ± 0.33, respectively. The regression coefficients for the linear portions of the two curves were not different ($p<0.05$).

3) Sensitivity

The sensitivity of ELISA was defined as the minimum amount of casein which may be measurable with accuracy and reliability. The mean and standard deviation for each set of three to five replicates were calculated, and the smallest concentration of casein which differed from zero by two standard deviations was defined as the limit of sensitivity for the assay. Various levels of sensitivity were achieved by varying the conditions of the assay. The minimum amount of casein detectable from the most sensitive assay was 2 ng/ml, while the maximum amount detectable from the least sensitive assay was 20 μg/ml. The parameter which affects the sensitivity of an assay the most, was the concentration of biotin-casein conjugate which competes for the binding sites of the antibody with the standard casein; the higher the concentration of the conjugate the less sensitive the assay. A 50% displacement (D_{50}) was defined as the amount of casein at which absorbance unit is a half of the maximum. The D_{50} was calculated from logistic fit program based on the method of Rodbard(1976) using Hewlett Packard 236/9,000 computer. The 50_{50s} achieved from various assays by adding the different concentration of biotinylated casein were approximately 20, 30, 90, 160, 400, 1,000, and 2,500 ng/ml and their corresponding biotinylated casein concentrations are 1, 2, 5, 50, 100, 500

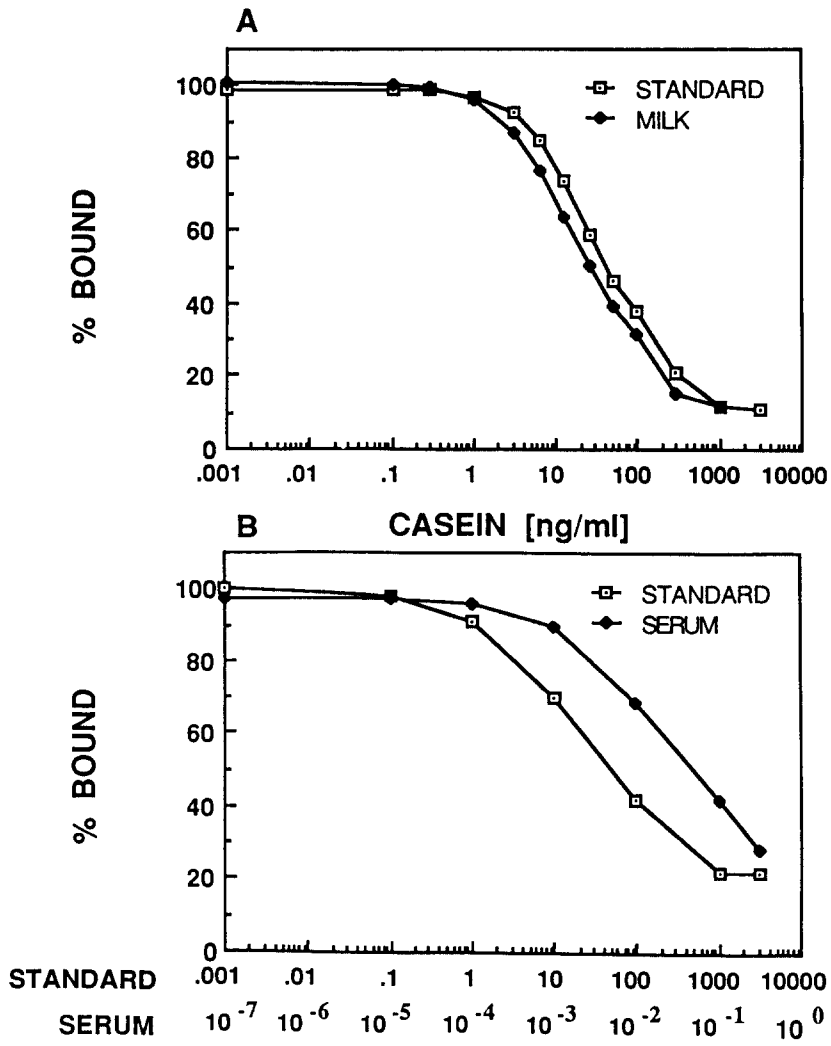

Fig. 4. Parallelism between A. reference standard and milk samples B. reference standard and serum sample dilutions. A. A parallelism between the reference standard and milk samples is shown at all concentration range. The slopes and their standard errors of the two curves were 1.24 ± 0.30 and 1.00 ± 0.22, respectively. The regression coefficients for the linear portions of the two curves were not different($p < 0.05$). B. A parallelism between the reference standard and serum sample dilutions is shown at all concentration range. The slopes and their standard errors of the two curves were 0.64 ± 0.24 and 0.74 ± 0.33, respectively. The regression coefficients for the linear portions of the two curves were not different($p < 0.05$).

and 1,000ng/ml. The summary of the standard curves with different sensitivities and the corresponding concentrations of biotinylated casein is listed in Table 1. Fig. 5 shows standard curves with various sensitivities and displays parallelism among standard preparations over various sensitivities. In general, higher sensitivity was achieved with a reduction of absorbance. The parameters studied to optimize the condition of the assay were concentration of biotinylated casein, incubation time, coating condition of antibody, pH and ionic strength of assay buffer,

Table 1. Summary of standard curves of casein ELISA with various sensitivities

n	Biotinylated Casein [ng/ml][1]	D_{50} [ng/ml]	Slope	Sensitivity [ng/ml]
5	1	19.5 (1.8)[2]	1.24 (0.30)	2.1 (.5)
3	2	29.9 (2.1)	1.17 (0.21)	4.2 (.9)
3	5	90.5 (16.7)	0.88 (0.34)	7.5 (1.3)
3	50	157.9 (15.5)	0.92 (0.18)	9.3 (2.1)
3	100	402.8 (36.5)	1.04 (0.32)	75.6 (10.5)
3	500	1004.6 (104.1)	1.22(0.31)	162.1 (21.3)
3	1000	2551.8 (386.1)	1.01 (0.33)	197.4 (34.7)

1. Biotinylated casein added.
2. Standard error.

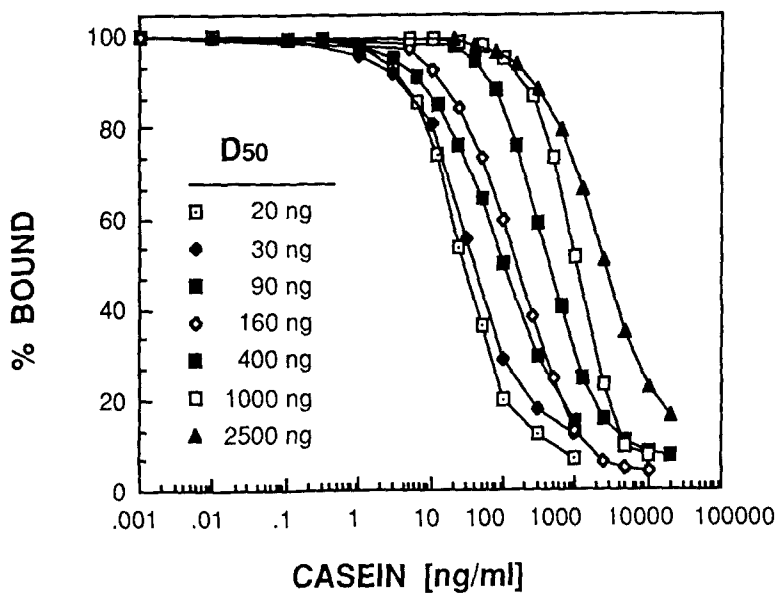

Fig. 5. Standard curves for bovine casein ELISA with various sensitivities, D_{50}'s at 20, 30, 90, 160, 400, 1000, 2500 ng/ml. The concentrations of biotinylated casein added for each curve were 1, 2, 5, 50, 100, 500, and 1000 ng/ml, respectively. The minimum amount of casein detectable from the most sensitive assay (D_{50} at 20 ng/ml) was 2 ng/ml, while the maximum amount from the least sensitive assay (D_{50} at 2500 ng/ml) was 20 μg/ml. Each point represents the mean of 5 replications.

Table 2. Possible problems in Casein ELISA and their causes and solutions

Problems	Causes	Solutions
High non-specific binging	Some preimmune Ab	Try another preimmune Ab.
	Impurity of Ab	Treat ethyl alcohol.
	Detergent	Increase detergent content.
	Non-Specific Ab	Give another booster shot.
		Use affinity chromatography.
Poor sensitivity	Too much casein-biotin	Add less casein-biotin.
	pH of substrate	Find optimal pH for enzyme.
	Poor Ab coating	Try different coating condition. (buffer, temperature, pH, incubation time, and microplate)
	Non-specific Ab	Give more booster shot. Check titer.
		Check Ab separation step.
Poor displacement	Low pH(<7.5)	Increase buffer pH(up to 9.0).
	High ionic strength	Lower ionic strength(<0.02M).
	Too much Ab	Coat less Ab.
Inconsistent results	Cross-reaction	Remove cross-reaction source (Ca++, BSA, Citrate etc.).
	Standard preparation	Prepare new standard.
Poor signals	Biotinylation	Obtain new biotin. Remove sucrose.
		Optimize reaction at pH 7.0~8.0.
	Low casein-biotin	Increase the conjugate.
	Avidin-Peroxidase	Obtain new batch. Dilute less.
	Incubation Time	Incubate longer.

and pH of substrate. Table 2 describes the parameters which may increase or decrease either the sensitivity or the signal in casein ELISA.

4) Intra- and Inter-Assay Variation

Intra-assay variation is defined as a coefficient of variation of the replication in the same assay. Inter-assay variation is defined as a coefficient of variation of estimates of concentrations of an unknown replicated in several successive assays. Table 3 shows the coefficients of variation of intra-assay and inter-assay at several concentrations suggesting that an estimation of sample would be estimated best from a point close to D_{50} at which the coefficient of variation is the smallest.

4. Assay Variables

Throughout the development of casein ELISA, many variables which affect the sensitivity of an assay were observed such as pH, ionic strength of the assay buffer, the presence

Table 3. Intra- and Inter-assay Variation of Standard and Samples

Source	Casein [ng /ml]	Intra-assay Coeff. Variation(%)	Inter-assay Coeff. Variation(%)
Standard	6.3	8.3	10.2
	25.0	5.5	9.7
	50.0	10.2	12.8
	100.0	13.6	14.8
Milk Sample	10.9	12.6	13.4
	27.2	5.7	10.4
	50.2	13.2	14.2
	108.6	11.4	12.1

of metal ions, temperature both incubation and storage of samples, incubation time, cross-reaction with a component of an assay, solubility of casein, alcohol treatment of chicken antibody, standard preparation, coating conditions and specificity of antibody. Some of the variables mentioned above were difficult to generalize for the optimal condition bacause they together affect the sensitivity of an assay by either decreasing or increasing the non-specific binding. However, Table 2 is directed for a possible modification if one want to change variables. The table was based on observations and comparisons from several hundred assays. It is helpful for controlling the sensitivity of an assay when one requires to change the range of detection in casein ELISA. The sensitivity of an assay was influenced the most by the concentration of the biotinylated casein among the many variables.

The order of addition of the assay components also influenced the sensitivity of an assay. For example, when, on IgY-coated plates, 65μl of standard was added first and immediately 65μl of 5 ng /ml biotinylated casein, the displacement was very poor. In other experiment, 125μl of standard was added first and the plate was incubated for 30 min at room temperature followed by washing and the addition of the same final concentration of biotinylated casein. The standard curve had Y_{max}=0.279 A, slope=0.501, and D_{50}=6.8 ng /ml. Another standard curve obtained by the addition of casein-biotin conjugate first, which was immediately followed by the addition of standard, had Y_{max}=0.409 A, slope=0.869, and D_{50}=90.5 ng /ml. Even though the assays were performed on the same plate under the same condition, the two curves were different($p<0.01$). The effects due to the simultaneous addition of components in casein ELISA were also studied. The assay was performed on the same plate under the same conditions. When standard and casein-biotin conjugate were added simultaneously, the sensitivity was decreased. The standard curve obtained by the addition of casein-biotin conjugate first, immediately followed by the addition of the reference standard, had Y_{max}=0.489, slope=0.721, and D_{50}=22.8 ng /ml. The standard curve obtained by the simultaneous addition of standard and casein-biotin conjugate had Y_{max}=0.407, slope=0.843, and D_{50}=87.4 ng /ml. The two curves were different($p<0.01$).

5. Casein Concentration of Periparturient Milk Samples

The profile of changes in percentage of total milk protein in mammary secretion at peripatum is shown in Fig. 6.A. From 20 to 4d prepartum, the percentage of total milk protein varied from 17 to 36% probalbly due to the leakage of blood proteins into the mammary glands and the reabsorption of mammary proteins into peripherial circulation. Additional variations were noticed around the time of parturition. The percentage decreased gradually to about 3% at 2d postpatum. The trend may be the result of the formation of tight junctions between the secretory cells, the accumulation of immunoglobulines, and the formation of milk proteins. The effect of prepartum sampling of mammary secretion was not examined in this study. The two cows under investigation showed similar results. As shown in Fig. 6.B, the concentration of casein(α and β) in mammary secretion peaked at around parturition reaching 35 mg /ml.

When the concentrations of casein in mam-

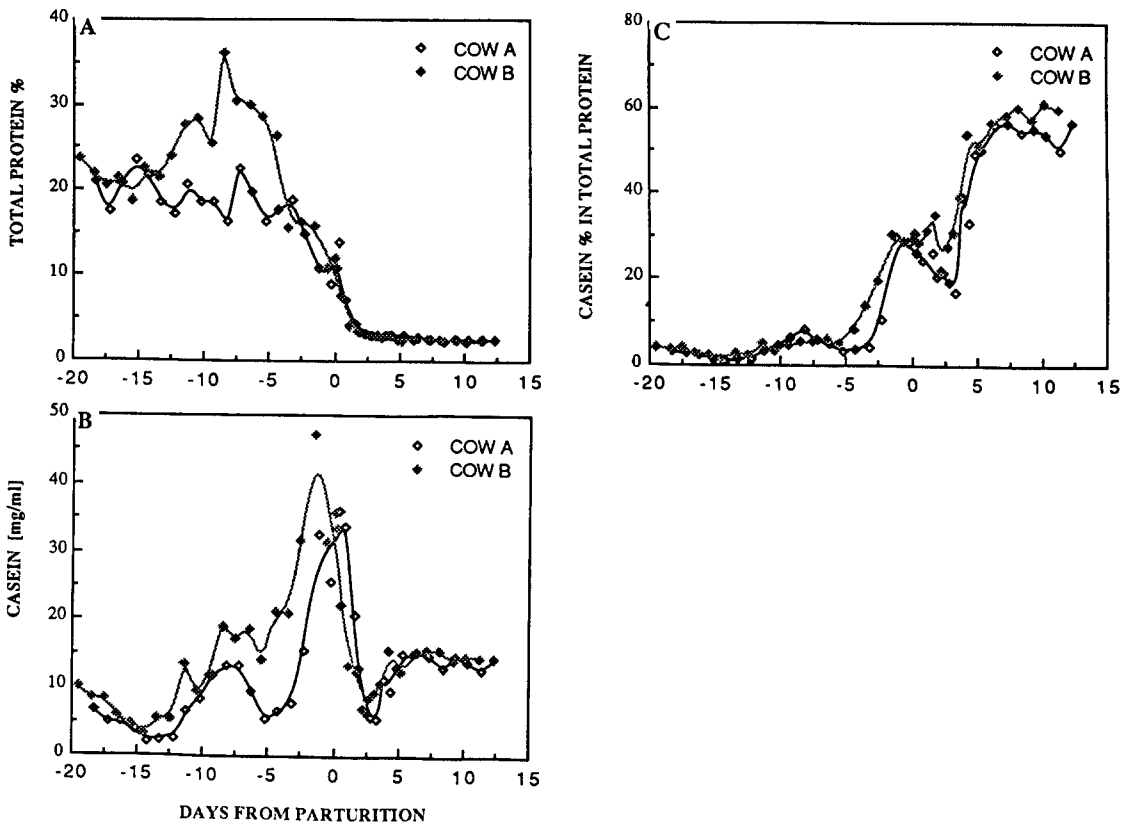

Fig. 6. A. Profile of changes in percentage of total milk protein in mammary secretion at peripartum. B. Profile of changes in concentration [mg/ml] of α-and β-casein in mammary secretion. C. Profile of changes in relative percentage of α-and β-casein in mammary secretion. Each point represents the mean of 3 independent experiments with triplication each.

mary secretions are expressed relative to the total protein, the relative changes in its synthesis can be approximated on the assumption that a relative fraction of a protein is constant. Fig. 6.C indicates the profile of the changes in relative percentage of casein in bovine mammary secretions. The percentage increased from less than 5% during most of prepartum period to 28% at parturition, and reached 57% at 7d postpartum. The profile of changes was different from that of alphalactalbumin in our previous report(Yom, 1987). This may suggest that the expression of the two milk proteins are regulated by different mechanisms.

Ⅳ. SUMMARY

A rapid, sensitive, and specific enzyme-linked immuno-sorbent assay(ELISA) for bovine casein was developed. Biotinylated casein and peroxidase-conjugated avidin were used in the assay with antibody separated from yolks of immunized hens. Caseins were biotinylated with sulfo-N-hydroxy succinimido biotin and peroxidase-conjugated avidin bound the biotinylated casein which became bound to immobilized antibody on a microplate. The antibodies were specific for bovine α- and β-caseins, and their cross-reactivities with whey proteins, IgG, and serum albumin from bovine were not detectable by ELISA and Western blot. Various sensitivities ranging from 2ng/ml to 20μg/ml of casein were achieved, and were controlled by adding various concentrations of the biotinylated casein. Parallelism was observed between standard and sample curves. The coefficients of variation of intra-assays and inter-assays from the most sensitive assay were 5.5 and 5.7%, respectively, at the 50% displacement. Casein contents of peripaturient milk samples showed that casein secretion rapidly increased 3d prepartum.

Ⅴ. CONCLUSIONS

A competitive type of casein ELISA was developed to estimate casein directly in bovine milk. The avidin-biotin system was sensitive and the chicken antibody was specific and sensitive enough to be used in an immunoassay. Various levels of sensitivity in casein ELISA were achieved and the sensitivity were controlled by adjusting concentration of biotinylated casein in an assay. The minimum casein detectable from the most sensitive assay was 2ng/ml, the maximum casein detectable from the least sensitive was 20μg/ml. The samples diluted to concentration of D_{50} allowed the minimum coefficient of variation. Casein ELISA with various sensitivities developed in this study can be employed as a useful tool for the quantitation of casein in milk and physiological studies *in vitro* for the effects of lactogenic hormones on the synthesis of casein, genetic studies such as selection of cows and bulls for casein and nutritional studies such as the effects of diets on milk proteins.

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