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Detection of α S1-casein in vomit from bottle-fed babies by enzyme-linked immunosorbent assay

Itaru Sato

Criminal Investigation Laboratory, Kanagawa Prefectural Police, 155, Yamashita-cho, Naka-ku, Yokohama, Japan

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Summary. This study describes a sensitive enzyme-linked immunosorbent assay (ELISA) using rabbit anti-bovine α S1-casein antibody for the detection of commercial milk and milk-containing vomit. The antibody does not react with other human body fluids such as breast milk. The stability of α S1-casein antigenic activity was examined after storage at different temperatures and enzyme digestion. There was no decrease after storage for one year at room temperature but 40% of the activity was lost after 6 months at 37°C. Enzyme digestion (6 hours, 37°C) resulted in 65~70% loss of activity but the antibody reacted with the peptide fragments of α S1-casein. Vomit samples from 3 normal infants were tested by ELISA, and α S1-casein could be detected in 1 cm^2 stain.

Key words: Forensic immunology – Infant – Asphyxiation – Aspiration pneumonia – Milk – Alpha S1-casein

Zusammenfassung. Die vorliegende Untersuchung beschreibt einen empfindlichen, enzymgebundenen Immunabsorptionstest (ELISA), der einen Anti-Rinder-α S1-Casein-Antikörper vom Kaninchen benutzt für den Nachweis kommerziell erhältlicher Milch und milchhaltigen Erbrochenem. Der Antikörper reagiert nicht mit anderen menschlichen Körperflüssigkeiten wie Brustmilch. Die Stabilität der Antigen-Aktivität von α S1-Casein wurde nach Lagerung bei verschiedenen Temperaturen und enzymatischem Verdau untersucht. Es gab keinen Abfall nach einjähriger Lagerung bei Raumtemperatur, aber 40% der Aktivität war nach sechsmonatiger Lagerung bei 37°C verloren. Enzymatischer Verdacht (6 Stunden, 37°C) führte zu einem Aktivitätsverlust von etwa 65–70%, aber der Antikörper reagiert mit den Peptid-Fragmenten von α S1-Casein. Proben von Erbrochenem von drei normalen Kindern wurde mit Hilfe der ELISA-Technik getestet und α S1-Casein konnte in 1 cm^2 großen Flecken nachgewiesen werden.

Schlüsselwörter: Forensische Immunologie – Säugling – Asphyxie – Aspirationspneumonie – Milch – Alpha S1-Kasein

Introduction

Sleeping infants sometimes aspirate their vomit accidentally, leading to a risk of death by aspiration pneumonia (Boulloche et al. 1986). Vomit stains can be useful for identifying the place where the victim asphyxiated since vomiting frequently occurs in such cases.

Breast milk can be identified by the microscopical presence of fat globules using Sudan Black staining, but this is not suitable for detecting modified milk powder because the membrane is ruptured by heating in the process of pasteurization (Eigel et al. 1984). Some classical chemical assays such as Moro, Umikoff and Matukura reactions have also been reported (Matukura 1940). Most of them, however, have some disadvantages in their practical applications in terms of sensitivity and organ specificity. Therefore, a more sensitive and specific method for the identification of modified milk powder is required.

α S1-casein is a major component of bovine milk (12~15 g/l) consisting of 199 amino acid residues (Mercier et al. 1971) but there is no corresponding protein in human milk (Otani et al. 1986a). In the present study, the stability and specificity of α S1-casein was examined by enzyme-linked immunosorbent assay (ELISA) using an antibody against α S1-casein to test its feasibility for the identification of bovine constituents in modified milk powder.

Materials and methods

Preparation of antibody against α S1-casein. Purified α S1-casein was obtained from bovine whole casein by the method of ion-exchange column chromatography described in a previous paper (Davies and Law 1977). The purity of this protein was checked by polyacrylamide gel electrophoresis (PAGE) using 4.5 *M* urea. α S1-casein (500 μg) was dissolved in 1 ml of 10 m*M* phosphate buffered saline (PBS), pH 7.2 and mixed with an equal volume of Freund's complete adjuvant. This was injected subcutaneously at several locations into the backs of rabbits and the serum was obtained 2 months later. Immunoglobulin G (IgG) was purified from the antiserum by affinity column chromatography using protein A-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) according

to the manufacture's instructions. Rabbit IgG was dialyzed against 100 m*M* phosphate buffer, pH 7.2 for more than 12 h at 4°C and stored at −80°C. Before use, the antibody was diluted with PBS to a protein concentration of 2 μg/ml.

Measurement of α S1-casein activity. α S1-casein antigenic activity was measured by two-step inhibition ELISA (Watanabe et al. 1983). Anti-α S1-casein antibody was mixed with dilutions of purified antigen or test sample in a test tube and incubated overnight at 4°C. Flat bottom wells of an ELISA plate (Sumitomo Bakelite co., Tokyo, Japan) were coated with 100 μl of purified α S1-casein (200 ng/ml) in 100 m*M* bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. Each well was washed 3 times with PBS containing 0.05% Tween 20 (T-PBS) before 200 μl PBS containing 1% bovine serum albumin (BSA) was added and incubated for 1 h at room temperature. After washing in T-PBS (×3), 100 μl of preincubated antigen-antibody mixture was added to the wells and incubated for 1 h at room temperature. The wells were washed with T-PBS (×3), and 100 μl of 800-fold diluted horseradish peroxidase (HRP) labelled anti-rabbit IgG goat serum (Tago Inc., CA, USA) was added. After incubation for 2 h at 37°C, the wells were washed with T-PBS (×5). The amount of α S1-casein antibody combined with the fixed antigen was estimated photometrically by the amount of HRP products (brown color) using the substrate o-phenylenediamine. To each well was added 100 μl of substrate solution (4.6 m*M* o-phenylenediamine in 100 m*M* Tris-HCl, pH 7.4 containing 0.03% H_2O_2). After incubation for 30 min at room temperature, the enzyme reaction was stopped by the addition of 100 μl of 2N sulfuric acid. Absorbance was measured at 490 nm by ImmunoReader NJ-2000 (Nippon Intermed co., Tokyo, Japan).

Protease digestion. Purified α S1-casein was digested by 0.5% pepsin in 10 m*M* HCl, pH 1.5, 0.5% trypsin or chymotrypsin in PBS for 6 h at 37°C. The reaction products were subjected to gel filtration performed on a Sephadex G-50 (Pharmacia LKB) column (1.5 × 100 cm) equilibrated with PBS at 4°C. The column was eluted at a flow rate of 24 ml/h and the eluents were collected in 3.5 ml fractions.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 17.5% acrylamide gel according to the procedure described by Laemmli (1970).

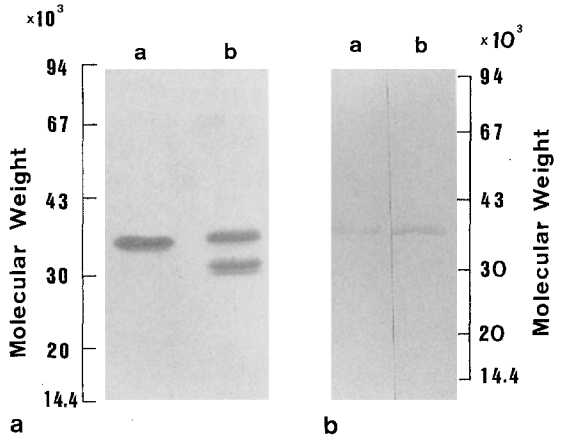

Fig. 1a SDS-PAGE electrophoretic patterns for the SDS soluble α S1-casein and bovine whole casein. Proteins separated on a 17.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue R 250. *lane a* purified α S1-casein; *Lane b* bovine whole casein. Arrows indicate α S1-casein, β-casein and κ-casein from top to bottom, respectively. **b** Identification of α S1-casein antigen by immunoblotting. After transfer to a nitrocellulose membrane, bands were detected with anti-α S1-casein antibody. *Lane a* bovine whole casein; *Lane b* purified α S1-casein

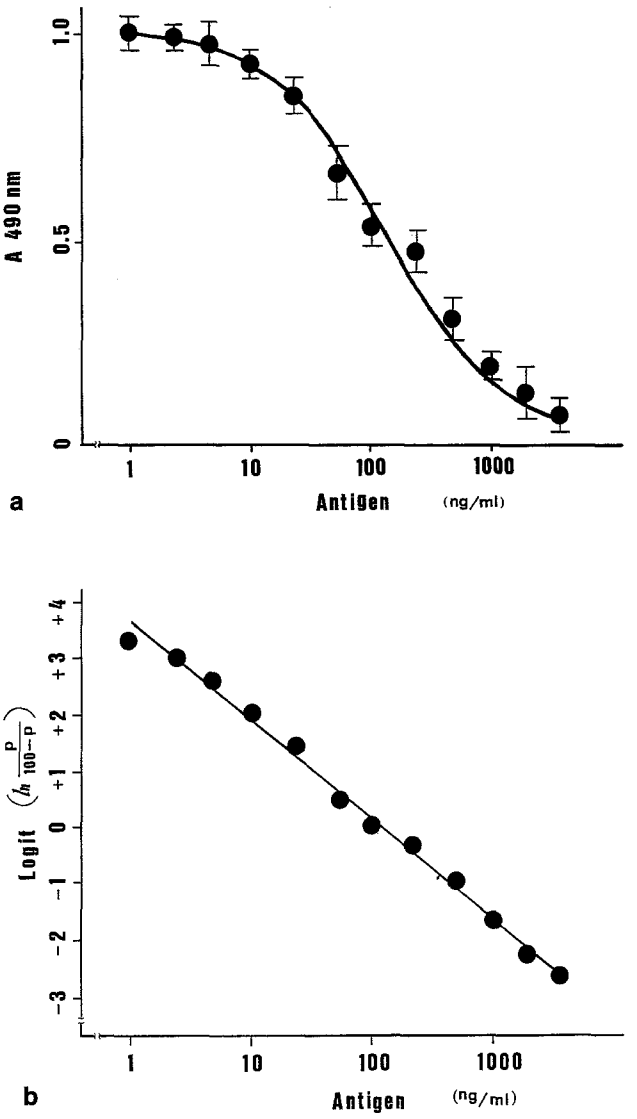

Fig. 2a Two-step inhibition pattern in ELISA. The vertical bars represent the standard deviation of 8 independent experiments. **b** Calibration curve for the measurement of α S1-casein. Absorbance was obtained from **a** and which was transformed by percentage (p). The solid symbols plot the resultant values after the various percentages were transformed by the formula of logit

Table 1. Cross-reactivity of human body fluids against anti-α S1-casein antibody

Antigen		Requisite protein concentration for 50% inhibition (μg/ml)	Relative activity (%)
α S1-casein		0.15	100
Human			
Serum	5[a]	250	0.6
Semen	5	>1000	<0.02
Breast milk	2	>1000	<0.02
Vaginal fluid	7	>1000	<0.02
Urine	5	>1000	<0.02
Saliva	5	>1000	<0.02

[a] Number of samples

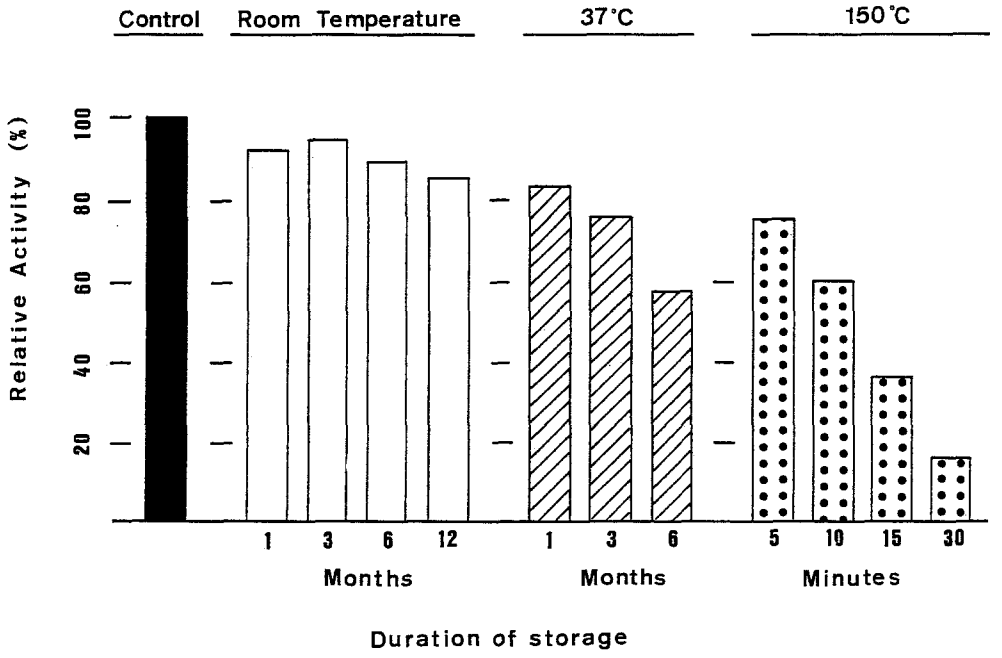

Fig. 3. Thermostability of α S1-casein

Transfer of proteins from a SDS-PAGE gel to a nitrocellulose membrane (0.45 μm pore size, Toyo Roshi co., Tokyo, Japan) was performed according to the method of Towbin et al. (1979). Anti-α S1-casein antibody (1 μg/ml) and HRP labelled anti-rabbit IgG goat serum (1:1000) were used for this assay, and the membrane was developed by incubation in the substrate solution (3 m*M* 4-chloro-1-naphthol in 50 m*M* Tris-HCl, pH 7.4 containing 0.03% H_2O_2). The total soluble protein content was determined by the method of Lowry et al. (1951), using BSA as a standard.

Table 2. Effect of protease digestion on antigenicity of α S1-casein

Treatment	Requisite concentration for 50% inhibition (μg/ml)	Relative activity (%)
None	0.15	100
Pepsin	0.57	26.32
Trypsin	0.74	20.27
Chymotrypsin	0.85	17.64

Results

Specificity of α S1-casein antibody against bovine whole casein

To examine whether the α S1-casein antibody reacted with other bovine caseins, bovine whole casein was electrophoresed by SDS-PAGE and analysed by immunoblotting assay. Purified α S1-casein showed a single band and whole casein showed more than 3 bands corresponding to α S1-casein. β-casein and κ-casein as shown in Fig. 1(a). These caseins have lower mobilities than those expected on the basis of their known molecular weights (α S1-casein, β-casein and κ-casein: 23600, 24000 and 19000, respectively), as described by Creamer and Richardson (1984). Figure 1(b) shows the results of immunoblotting with anti-α S1-casein antibody with a single positive band of immunoreactive α S1-casein corresponding to purified α S1-casein.

Quantitative determination of α S1-casein by two-step inhibition ELISA

The antibody was first reacted with the antigen in a test tube and then with purified α S1-casein precoated on an ELISA plate. A standard curve was obtained using standard samples of purified α S1-casein and the concentration was plotted against absorbance at 490 nm (Fig. 2a). The curve was transformed to a straight line by logit (Fig. 2b). When no antigen was present the total amount of antibody which bound the precoated antigen achieved the maximum (100%) whereas 150 ng/ml of antigen lowered the amount of the bound antibody to 50%. The minimum and maximum limits of the assay were fixed at 5 ng/ml and 40 μg/ml of antigen, respectively. Using this calibration curve, the concentration of α S1-casein in commercial milk powder (6 brands from 2 companies in Japan) was determined as 138.7 ± 24.3 (mean ± S.D.) μg/ml.

Detection of α S1-casein in various human body fluids

To clarify whether human body fluids contained α S1-casein antigenic activity, human serum, semen, breast milk, vaginal fluid, urine and saliva were examined by ELISA. Cross-reactivity was determined as the protein concentrations of body fluids which gave 50% inhibition. As shown in Table 1 250 μg/ml of human serum, a 1000-fold dilution of purified α S1-casein, was required to achieve 50% inhibition. Human semen, breast milk, vaginal fluid, urine and saliva did not show any inhibition even at a concentration of 1 mg/ml.

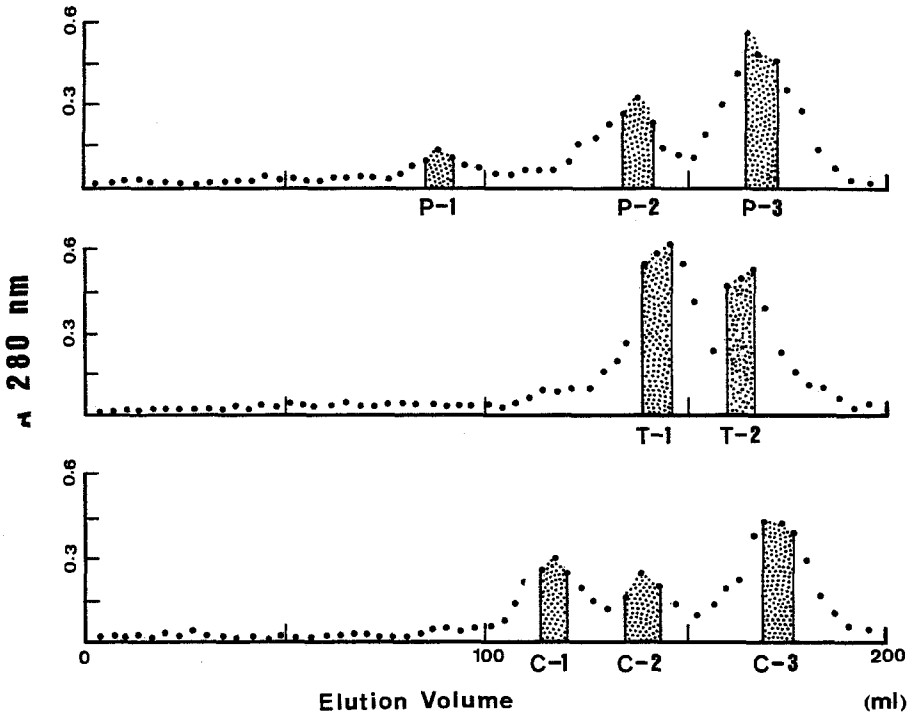

Fig. 4. Gel filtration α S1-casein digested with proteases on Sephadex G-50 column. P, T and C indicate pepsin, trypsin and chymotrypsin digestions, respectively

Table 3. Antigenic activity of peptide fragments of αS1-casein after protease digestion

Treatment	No. of peak fraction[a]	Relative activity[b]
Pepsin	P-1	59.7
	P-2	11.4
	P-3	3.0
Trypsin	T-1	50.8
	T-2	3.9
Chymotrypsin	C-1	46.5
	C-2	24.9
	C-3	1.1

[a] Purified αS1-casein was digested with pepsin, trypsin or chymotrypsin. After gel filtration of resultant peptide fragments on Sephadex G-50 column, the peak fractions were collected as shown in Fig. 4

[b] Relative antigenic activity of peptides obtained by gel filtration against the activity of original proteins

Table 4. Detection of immunoreactive αS1-casein in vomit stains

Case no.	Source	Post suckling time	αS1-casein[a] (ng/mg)	Protein concentration (μg/ml)
1	Bovine	Within 0.5 h	120	510
2	Bovine + human	Within 0.5 h	42	200
3	Bovine + human	Within 0.5 h	73	420
4	Bovine	Within 0.5 h	197	230
5	Bovine	1~1.5 h	331	325
6	Human	1~1.5 h	0	180
7	Bovine + human	1.5~2 h	292	465
8	Bovine	1~2 h	120	250
9	Bovine	About 3 h	63	170

[a] Concentration of αS1-casein or its peptides estimated from calibration curve of ELISA

Stability of the antigenic activity of α S1-casein

Purified α S1-casein (10 mg) was stored at 3 different temperatures to evaluate the stability of antigenic activity (Fig. 3). When stored at room temperature no change in activity was observed even after one year. When stored at 37°C under humid conditions, the activity decreased 17% in 3 months and 42% in 6 months. At 150°C, 80% of activity was lost after only 30 min.

To examine whether antigenic activity of α S1-casein is resistant to proteolytic digestion, α S1-casein was treated with pepsin, trypsin or chymotrypsin. Approximately 26%, 20% and 17% activity remained after treatment with pepsin, trypsin or chymotrypsin, respectively (Table 2). The antigenic activity of the resultant peptides was examined by applying to a Sephadex G-50 column. Figure 4 shows the typical elution patterns of α S1-casein cleaved with pepsin, trypsin or chymotrypsin. Distinct peaks were obtained after pepsin (3), trypsin (2) or chymotrypsin (3) digestion. Each peak was pooled and concentrated using a Stirred Ultrafiltration Cell (Model 8010, Amicon co., Mass, USA) and examined for antigenic activity by ELISA. In all cases, high molecular weight chains showed higher activity than low molecular weight chains (Table 3).

Detection of immunoreactive α S1-casein in vomit

To clarify whether forensic samples contained commercial milk, vomit samples from 3 babies aged 2~6 months

who had been breast and bottle-fed or only bottle-fed, were examined. These samples were collected on cotton, air dried and then applied to the quantitative detection of α S1-casein by ELISA. Cotton stains with vomit (1 cm^2) were extracted in 250 µl of PBS, and 100 µl of the supernatant was used for the determination of protein concentration. The extract (100 µl) was diluted 10-fold, 100-fold or 1000-fold with PBS and the α S1-casein was measured by ELISA using the calibration curve (Table 4). α S1-casein could always be detected except in vomited breast milk. Antigenic activity could be detected 3 h after suckling.

Discussion

In the present study the high specificity and stability of the antigenic activity of purified α S1-casein was demonstrated. These results indicate that α S1-casein may be a good marker to identify bovine milk. α S1-casein was not detected in other human body fluids by ELISA. Furthermore, by immunoblotting assay it was found that the anti-α S1-casein antibody did not react with β-casein or κ-casein indicating that α S1-casein is specific for bovine milk. Antigenic activity could also be detected in the fragments of α S1-casein after proteolytic digestion indicating that the polyclonal antibody against α S1-casein could recognize fragments of α S1-casein (Table 3). This result is consistent with the fact that α S1-casein has at least 6 antigenic epitopes (Otani et al. 1985) which exist on the primary structure of the amino acid sequence (Otani et al. 1986b). Therefore, α S1-casein could be identified from digested bovine milk which had been regurgitated from the stomach into the respiratory tract. Preliminary experiments showed that α S1-casein could be detected in vomit where the proteins have been digested.

In addition, it should be stressed that even after storage for 6 months at 37°C, half of the activity remained.

The ELISA system used in this study could be very useful in forensic science because of its high sensitivity (5 ng/ml of α S1-casein could be detected). The only disadvantage of this ELISA system lies in its complicated procedure but if α S1-casein is used a simple procedure such as dot immunoassay is recommended.

References

Boulloche J, Mallet E, Basuyau JP, Tayot P, Samson-Dollfus D (1986) The value of serum IgE assay in milk aspiration and the sudden infant death syndrome. Acta Paediatr Scand 75:530–533

Creamer LK, Richardson T (1984) Anomalous behaviour of bovine α S1- and β-caseins on gel electrophoresis in sodium dodecyl sulfate buffers. Arch Biochem Biophys 234:476–486

Davies DT, Law AJR (1977) An improved method for the quantitative fractionation of casein mixtures using ion-exchange chromatography. J Dairy Res 44:213–221

Eigel WN, Butler JE, Ernstrom CA, Farrell HM, Harwalkar VR, Jenness R, Whitney R McL (1984) Nomenclature of proteins of cow's milk, J Dairy Sci 67:1599–1631

Lammli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Matukura T (1940) Die Anwendung der Milchkoagulationsreaktion zur Unterscheidung von Menschen- und Kuhmilch in der gerichtlichen Medizin (in Japanese, abstract in German) Acta Crim Japon 14:11–20

Mercier JC, Grosclaude F, Ribadeau-Dumas B (1971) Structure primarie de la caseine α S1-bovine. Eur J Biochem 23:41–46

Otani H, Takayama K, Tokita F (1985) Antigenic identity of bovine α S1- and α S1-1 caseins. Milchwissenschaft 40:24–26

Otani H, Takahashi F, Tokita F (1986a) Antigenic reactivities of fragments obtained by cyanogen bromide cleavage of bovine α S1-casein toward antiserum to intact α S1-casein. Agric Biol Chem 50:607–613

Otani H, Takayama K, Tokita F (1986b) Studies on the antigenicity of bovine α S1-casein: antigenic activities of some peptides derived from C-terminal region. Milchwissenschaft 41:565–568

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Watanabe S, Dinh TL, Ochiai Y, Hashimoto K (1983) Immunochemical specificity of myosin light chains from mackerel ordinary and dark muscles. J Biochem 94:1409–1419