

# Electrochemical Sensing of Casein Based on the Interaction between Its Phosphate Groups and a Ruthenium(III) Complex

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A reaction to casein, along with  $\beta$ -lactoglobulin, is a main cause of milk allergies, and also is a useful indicator of protein in allergic analyses. In the present study, a simple casein sensor was developed based on the interaction between a phosphate group of casein and electroactive  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ . We evaluated the voltammetric behavior of a casein- $[\text{Ru}(\text{NH}_3)_6]^{3+}$  complex using a glassy carbon electrode. When the ruthenium(III) complex was combined with the phosphate groups of casein, the structure of the casein was changed. Since the hydrophobicity of casein was increased due to the binding, the casein was adsorbed onto the electrode. Furthermore, we modified an electrode with a ruthenium(III) ions/collagen film. When the sensor was applied to the detection of the casein contained in milk, the values coincided with those indicated by the manufacturer. Accordingly, this electrode could be a powerful sensor for the determination of casein in several foods.

**Keywords** Casein, hexaammineruthenium(III) ions, collagen film, phosphate group, allergen

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## Introduction

The number of patients with allergic diseases caused by foods continues to increase.<sup>1,2</sup> Food allergies are caused by undigested protein (peptide) fragments. Because the proteins (peptides) contain a protease-resistant domain, they are recognized as foreign substances.<sup>3,4</sup> As a result, IgE antibodies are produced that specifically react to the domain, and histamines based on the allergic reaction are released.<sup>5,6</sup> Items found in 25 foods that trigger allergies have been established by the Ministry of Health, Labor and Welfare in Japan.<sup>7</sup> Seven of those items in foods (egg, milk, wheat, peanut, prawns, soba and crab) have elicited severe symptoms, and are classified as “specific raw foods” and mandatory advisory information is circulated. The damaging effect of an allergy to eggs is the most frequent in infants in a number of countries.<sup>8</sup> Accordingly, screening for egg allergies is required for control of the allergy symptoms.<sup>9</sup> Milk is the food allergen that ranks second in prominence to eggs, and also is common in young children.<sup>10</sup> Milk protein consists of milk serum protein (20%) and casein (80%).<sup>11</sup> Casein is an allergen in cow’s milk along with  $\beta$ -lactoglobulin, and is an important indicator of protein in the allergic analysis of milk.<sup>12</sup> Casein is one of the phosphorylated proteins with amphipathic properties that inhibit an orderly structure due to the existence of proline-rich proteins.<sup>13–15</sup> In the unique properties of the casein, the hydrophilic moiety of casein involves phosphorylated serine residues that have a high affinity for calcium ions.<sup>16,17</sup> The interaction caused change in the structure of the casein to be

aggregated.  $\kappa$ -Casein has sugar chains, however, and the aggregation was suppressed.

Many methods to detect casein have been proposed in order to monitor this allergen in foods. For example, detection of casein was attempted using surface plasmon resonance immunosensor.<sup>18</sup> Casein has also been detected using enzyme-linked immune sorbent assay (ELISA),<sup>19</sup> sodium dodecyl sulfate-polyacrylamide gel electrophoresis,<sup>20</sup> western blot,<sup>21</sup> and microarray analyses.<sup>22</sup> In addition, casein fractions have also been separated using C-8 reverse-phase and diethylaminoethyl-type anion-exchange HPLC systems.<sup>23</sup> Cao *et al.* reported an electrochemical immunosensor of casein using gold nanoparticles and a poly(L-arginine)/multi-walled carbon nanotubes composite film.<sup>24</sup> Our group suggested a voltammetric sensing of ovalbumin using an electrode modified with gallium(III),<sup>25</sup> but an interaction with gallium(III) caused ovalbumin to accumulate on the electrode. However, the electrode response of the gallium(III) ion was not obtained when the complex between gallium(III) and ovalbumin was formed. To electrochemically measure the ovalbumin, special marker ions were needed. We knew that DNA had been detected using the electrostatic interaction between the anionic phosphate groups of DNA and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ .<sup>26</sup> Therefore, the sensing of ovalbumin using a ruthenium(III) complex combined with the phosphate group of ovalbumin was performed. The change in the electrode response was based on the competitive reaction between gallium(III) and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  to the phosphate groups. The system showed high selectivity for ovalbumin, and the electrode response of  $2.0 \times 10^{-9}$  M ovalbumin was 20-fold that of  $2.0 \times 10^{-9}$  M  $\alpha$ -casein. This difference in sensitivity may have been due to the structure change of the protein caused from gallium(III) and ruthenium(III).

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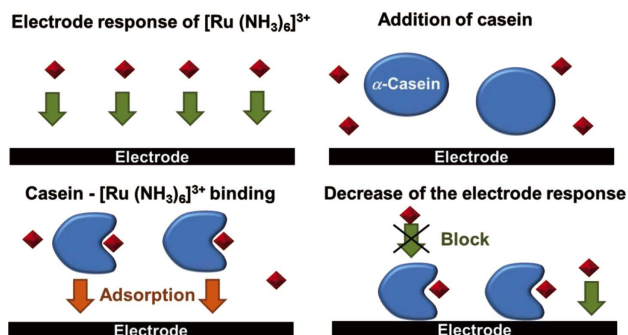


Fig. 1 Principle of casein detection using  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  on a glassy carbon electrode.

In the present study, an electrochemical sensing of casein based on an interaction between its phosphate groups and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was performed. First, the casein was measured *via* the binding between  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and casein using a bare glassy electrode (Fig. 1). Ono reported that the hydrophobicity of the calcium-casein complex was greater than that of casein alone.<sup>27</sup> When this phenomenon was observed in the formation of a casein- $[\text{Ru}(\text{NH}_3)_6]^{3+}$  complex, a stronger adsorption of the complex on the electrode is expected. The selective detection of casein is achieved using suppression of the electrode response of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  caused by the adsorption of the casein on the electrode surface. The merit of this procedure is that a competitive reaction against casein between  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and gallium(III)-modified electrode mentioned above is not needed. The detection is performed by simply using the change in the electrode response based on the binding between casein and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ . Therefore, an electrode modified with ruthenium(III) ions/collagen film was constructed on the basis of results obtained using a bare electrode (Fig. 2). The ruthenium(III) was immobilized on the collagen film using *N*-[5-(4-isothiocyanatobenzyl)amido-1-carboxypentyl] iminodiacetic acid (isothiocyanatobenzyl-nitrilotriacetic acid (NTA)).<sup>28</sup> The principle of this casein detection is that the peak current of ruthenium(III) immobilized on the electrode surface is changed due to the binding between casein and ruthenium(III). Furthermore, the ruthenium(III)-modified electrode was applied to the detection of the casein contained in the milk in order to examine the function of the electrode.

## Experimental

### Reagents

Hexaammineruthenium(III) chloride,  $\alpha$ -casein,  $\alpha$ -casein dephosphorylated from bovine milk ( $\geq 70\%$   $\alpha$ -casein basis),  $\beta$ -casein,  $\kappa$ -casein, concanavalin A (Con A), bovine serum albumin (BSA), and ovalbumin (OVA) were purchased from Sigma-Aldrich. A ruthenium(III) standard solution, 1000 ppm, was obtained from Merck. Isothiocyanatobenzyl-NTA and 2-(5-bromo-2-pyridylazo)-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino] phenol, disodium salt, dehydrate (5-Br-PAPS) were supplied by Dojindo. The collagen solution (I-PC 5 mg/mL) was purchased from Koken. The molecular weight of the collagen solution was 300000, and the collagen contained arginine and lysine residues (4.99 and 2.59%). A phosphate buffer (0.1 M) with  $\text{KH}_2\text{PO}_4$  (0.1 M) and NaOH (0.1 M) was used for the voltammetric measurements. All reagents used were of analytical reagent grade.

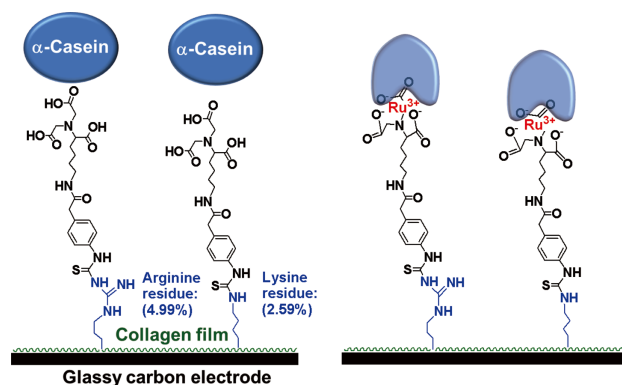


Fig. 2 Principle of casein detection using an electrode modified with ruthenium(III) complexes.

### Apparatus

Voltammetric measurements were carried out using an ALS electrochemical analyzer Model 612B. The working electrode was a glassy carbon version (diameter, 3.0 mm, Model No. 002012, BAS). On the other hand, a glassy carbon electrode with the plate ( $2 \times 25 \times 25$  mm, Model No. 12087, BAS) cut in half was mounted on a plate material-evaluating cell (Model No. 11951, BAS) to construct an electrode modified with ruthenium(III)/collagen film. The electrode was polished using 1.0-, 0.3- and 0.05- $\mu\text{m}$  alumina (Baikowski International Corp., Charlotte, NC). An Ag/AgCl type (sat. NaCl, Model No. 012167, BAS) was the reference electrode and a platinum wire was the counter electrode. All potentials were measured against the Ag/AgCl electrode.

### Preparation of an electrode with ruthenium(III) ions/collagen film

The electrode modified with ruthenium(III) ions/collagen film was prepared as follows. First, the electrode was set on the plate of a material-evaluating cell. The collagen solution (10  $\mu\text{L}$ ) diluted to  $2.5 \times 10^{-3}$  g  $\text{mL}^{-1}$  using 0.1 M HCl was cast on the electrode and was dried for 60 min at room temperature. Next, the electrode was rinsed three times with 0.1 M bicine buffer. Then, 0.02 M of isothiocyanatobenzyl-NTA was immobilized to the amino group of the arginine and lysine residues of the collagen film surface in 0.1 M bicine buffer at pH 8.5 (40  $\mu\text{L}$ ) with dimethyl sulfoxide (10  $\mu\text{L}$ ) for 60 min at 37°C. The solution was removed, and the plate was rinsed three times with 0.1 M  $\text{NaHCO}_3$  and 0.1 M HCl. Then, 500 ppm of ruthenium(III) in 50  $\mu\text{L}$  of 0.1 M  $\text{NaHCO}_3$  (pH 7.0) with 0.1 M HCl was mixed using a nitrogen purge to react with the NTA for 60 min. The amount of the immobilized ruthenium(III) was estimated based on the difference in absorbance by 615 nm of free ruthenium(III) in a solution before and after immobilization using 5 Br-PAPS.<sup>29</sup> After  $5.0 \times 10^{-3}$  M 5-Br-PAPS and the solution with ruthenium(III) ions with 10% ethylene glycol in 0.1 acetate buffer (pH 5.2) were incubated at 95°C for 30 min, the spectrometric measurements were carried out at 25°C.

### Voltammetric measurements of ruthenium(III) complex with casein

Voltammetric measurements of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and ruthenium(III) ions were performed using cyclic voltammetry (CV), linear sweep voltammetry (LSV) (scan rate, 50  $\text{mV s}^{-1}$ ), and differential pulse voltammetry (DPV) (scan rate, 5  $\text{mV s}^{-1}$ ; pulse amplitude, 50 mV; sample width, 2 ms; pulse width,

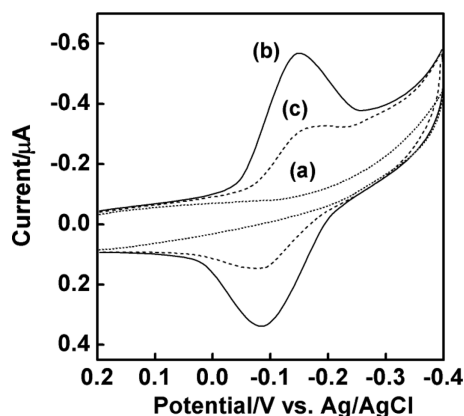


Fig. 3 Cyclic voltammograms of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein using a glassy carbon electrode. (a) Blank; (b)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ; (c)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M  $\alpha$ -casein.  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). Measurements were performed using linear sweep voltammetry (scan rate,  $50 \text{ mV s}^{-1}$ ) after a potential of 0.2 V was applied to the electrode for 5 min with stirring.

50 ms; and, pulse period, 200 ms) in 0.1 M acetate buffer (pH 3.2). The solution was de-aerated for 10 min using a nitrogen purge before the voltammetric measurements. Voltammograms of  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  were recorded and carried out in the range of from 0.2 to  $-0.4$  V after a potential of 0.2 V for 5 min was applied to a glassy carbon electrode. For the measurements with casein, the potential was scanned after  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M protein were incubated for 10 min in 0.1 M of acetate buffer (pH 3.2). The other conditions were the same as those without casein. The detection of casein using an electrode modified with ruthenium(III) ions was carried out *via* DPV. The potential was scanned in a positive direction ranging between 0.2 and  $-0.4$  V. The procedure based on the steps mentioned above was attempted using DPV.

## Results and Discussion

### Voltammetric measurements of hexaammineruthenium(III) ions with $\alpha$ -casein

To examine the interaction between  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein, cyclic voltammograms were recorded using a glassy carbon electrode. When  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was added to 0.1 M acetate buffer (pH 3.2), the potential was scanned in a range of between 0.2 and  $-0.4$  V after the potential of 0.2 V for 5 min was applied to the electrode (Fig. 3). The oxidation and reduction peaks of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  appeared at  $-0.08$  and  $-0.16$  V. Next,  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $3.0 \times 10^{-8}$  M  $\alpha$ -casein were incubated for 10 min with stirring. Then, a voltammetric measurement was carried out under the same conditions. As a result, the reduction peak was decreased to 50% that of  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  alone. However, the change in the oxidation peak was smaller than that of the reduction peak. Therefore, the reduction peak was used to evaluate the voltammetric behavior of  $\alpha$ -casein. The relationships between the peak current and the time with a potential are shown in Fig. S1 (Supporting Information). The electrode response of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  without  $\alpha$ -casein was independent of the time. In the presence of  $\alpha$ -casein, the peak current was constant for more than 4 min. For the formation of the casein-ruthenium(III)

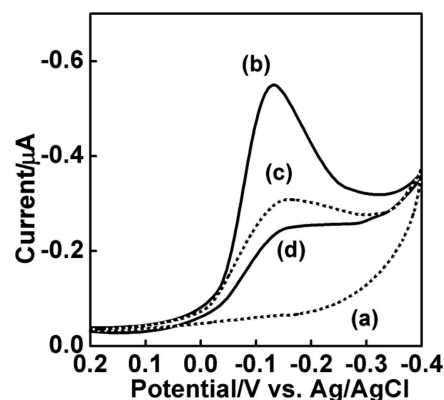


Fig. 4 Linear sweep voltammograms of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with calcium(II) using a glassy carbon electrode. (a) Blank; (b)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ; (c)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M  $\alpha$ -casein; (d)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M  $\alpha$ -casein +  $3.0 \times 10^{-5}$  M calcium(II). The procedure for (c):  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). The procedure for (d):  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ,  $\alpha$ -casein, and calcium(II) were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). Measurements were performed using linear sweep voltammetry (scan rate,  $50 \text{ mV s}^{-1}$ ) after a potential of 0.2 V was applied to the electrode for 5 min with stirring.

complex, the time mentioned above was needed. When the binding between  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein occurred in the solution, the concentration of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was 1000-fold that of  $\alpha$ -casein. The  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  functioned as the ligand and marker ions against casein. The difference in the concentration indicated that the binding was not based on the stoichiometrical reaction. When  $3.0 \times 10^{-5}$  M of calcium(II) was added to the solution with  $\alpha$ -casein and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ , however, the electrode response of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was repressed in the presence of the calcium(II) (Fig. 4). Although the peak current of  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with  $\alpha$ -casein was 53% that of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  alone, the peak current with  $\alpha$ -casein and  $3.0 \times 10^{-5}$  M calcium(II) was 38%. The decrease of the peak current due to the formation of a casein-ruthenium(III) complex was greater than that of a casein-calcium(II) complex. Calcium(II) has a high affinity for  $\alpha$ -casein and causes a conformational change in  $\alpha$ -casein.<sup>27</sup> Since the binding produces an increase in the hydrophobicity of  $\alpha$ -casein, the  $\alpha$ -casein strongly adsorbs onto the electrode. The interaction between  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein might be the same as the effect produced from the formation of  $\alpha$ -casein-calcium(II) complex. Because the electrode surface was covered with  $\alpha$ -casein with  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ , the electrode response of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  decreased as the concentration of  $\alpha$ -casein increased.

### Effect of phosphate groups to the peak current of $[\text{Ru}(\text{NH}_3)_6]^{3+}$

The binding between  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and a protein may be affected by the number of phosphate groups in the protein. The number of phosphate groups in casein and other proteins is listed in Table 1. The numbers per mole in  $\alpha$ -casein,  $\beta$ -casein, and  $\kappa$ -casein were 8–10, 4–5, and 1, respectively.<sup>30,31</sup> Ovalbumin has a phosphate group per mol.<sup>32</sup> The measurements were carried out using DPV, and the procedure was similar to that shown in Fig. 7. The voltammograms of  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  were recorded after a potential of 0.2 V was applied for 5 min to a glassy carbon electrode. Then, the potential was scanned after  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M protein were incubated for 10 min in 0.1 M of acetate

Table 1 Ratio of the peak current of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with a protein to the peak current of only  $[\text{Ru}(\text{NH}_3)_6]^{3+}$

| Protein                                       | The number of phosphate groups | Ratio, <sup>a</sup> % |
|-----------------------------------------------|--------------------------------|-----------------------|
| $\alpha$ -Casein                              | 8 – 10                         | 53                    |
| Dephosphorylated $\alpha$ -casein $\geq 70\%$ | $\alpha$ -Casein basis         | 85                    |
| $\beta$ -Casein                               | 4 – 5                          | 43                    |
| $\kappa$ -Casein                              | 1                              | 89                    |
| OVA                                           | 1                              | 92                    |
| BSA                                           | 0                              | 87                    |
| Con A                                         | 0                              | 96                    |
| SBA                                           | 0                              | 99                    |
| WGA                                           | 0                              | 98                    |

$[\text{Ru}(\text{NH}_3)_6]^{3+}$  and protein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). Measurements were carried out using differential pulse voltammetry after a potential of 0.2 V was applied to the electrode for 5 min with stirring.

$$a. \text{ Ratio} = \frac{I_p \text{ of } 3.0 \times 10^{-5} \text{ M } [\text{Ru}(\text{NH}_3)_6]^{3+} \text{ with } 3.0 \times 10^{-8} \text{ M protein}}{I_p \text{ of only } 3.0 \times 10^{-5} \text{ M } [\text{Ru}(\text{NH}_3)_6]^{3+}}$$

buffer (pH 3.2). To evaluate the effect of the groups, the ratio of the peak currents was defined as follows: Ratio = ( $I_p$  of  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with  $3.0 \times 10^{-8}$  M protein)/( $I_p$  of only  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ). The ratio of the peak current in a solution with  $\beta$ -casein was similar to that of  $\alpha$ -casein. When  $3.0 \times 10^{-8}$  M dephosphorylated  $\alpha$ -casein was added to the solution, the peak current of  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was approximately 85% that without the casein (Fig. 5). The percentage of the dephosphorylation was more than 70%. As a result, the electrode response with the dephosphorylated  $\alpha$ -casein was greater than that of the phosphate group that was rich in  $\alpha$ -casein. The phenomenon indicated that the interaction between the dephosphorylated  $\alpha$ -casein and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was weaker. Based on the number of phosphate groups, the decrease of electrode response with  $\kappa$ -casein was less than that with  $\alpha$ - and  $\beta$ -caseins. The peak current of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with ovalbumin combined with a phosphate group was 92% that without ovalbumin. This was because of the difference in the number of phosphate groups between  $\alpha$ -casein and ovalbumin. The peak currents of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with the simple proteins of BSA, Con A, SBA, and WGA were measured using the same procedure. A decrease in the electrode response rarely appeared in Con A, SBA, or WGA without the phosphate group. However, the peak current with BSA was influenced by the adsorption of BSA onto the electrode. As a result, the  $\alpha$ -casein and  $\beta$ -casein with more phosphate groups had an advantage in the formation of a complex. It seems that the change in the structure of casein was related to the binding between casein and ruthenium(III).

#### Electrode response of hexacyanoferrate and hydroquinone with $\alpha$ -casein

To investigate whether  $\alpha$ -casein had selectively combined with  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ , voltammetric measurements of other electroactive marker ions were performed in its presence. Figure 6(A) shows the cyclic voltammograms of negatively-charged  $[\text{Fe}(\text{CN})_6]^{3-}$  in 0.1 M acetate buffer. When the potential was swept in the negative direction, a reduction peak of 0.16 V was observed at the glassy carbon electrode. The oxidation peak of  $[\text{Fe}(\text{CN})_6]^{3-}$  was obtained at 0.25 V. Next, the peak of  $3.0 \times 10^{-5}$  M  $[\text{Fe}(\text{CN})_6]^{3-}$  was measured after a stirring of the solution with  $3.0 \times 10^{-8}$  M  $\alpha$ -casein for 10 min. Because the peak current was rarely changed by the coexistence of casein,

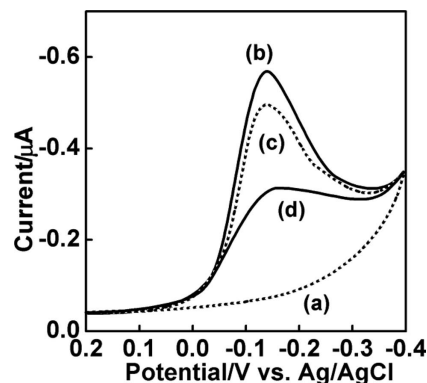


Fig. 5 Linear sweep voltammograms of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with dephosphorylated  $\alpha$ -casein using a glassy carbon electrode. (a) Blank; (b)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ; (c)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M dephosphorylated  $\alpha$ -casein; (d)  $3.0 \times 10^{-5}$  M  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  +  $3.0 \times 10^{-8}$  M  $\alpha$ -casein. The procedure for (c):  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and dephosphorylated  $\alpha$ -casein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). The procedure for (d):  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  and  $\alpha$ -casein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). Measurements were performed using linear sweep voltammetry (scan rate,  $50 \text{ mV s}^{-1}$ ) after a potential of 0.2 V was applied to the electrode for 5 min with stirring.

the binding interaction between  $\alpha$ -casein and  $[\text{Fe}(\text{CN})_6]^{3-}$  was smaller than that between  $\alpha$ -casein and  $[\text{Ru}(\text{NH}_3)_6]^{3+}$ . On the other hand, a cyclic voltammogram of  $3.0 \times 10^{-5}$  M hydroquinone was recorded in the range of 0.8 to  $-0.4$  V (Fig. 6(B)). A reduction peak due to the electrode reaction from quinone moieties to hydroxyl groups appeared at  $-0.18$  V. In contrast, an oxidation peak based on the opposite reaction appeared at 0.35 V. The voltammograms of hydroquinone were similar to those with  $3.0 \times 10^{-8}$  M  $\alpha$ -casein. These results show that the decrease in the peak current of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  was due to its complex with casein.

#### A casein sensor based on a ruthenium(III)-modified electrode with collagen film

We constructed an electrode modified with ruthenium(III) ions/collagen film based on the results obtained by a bare glassy carbon electrode. A glassy carbon plate (electrode area:  $0.196 \text{ cm}^2$ ) was coated with  $8.3 \times 10^{-11}$  mol of collagen film that contained  $1.4 \times 10^{-8}$  mol of residue made up of arginine and lysine amino groups.<sup>24</sup> Next, the immobilized amount of ruthenium(III) was estimated using 5-Br-PAPS after isothiocyanobenzyl-NTA was bound to the amino groups in the collagen film. The value of the ruthenium(III) ( $7.2 \times 10^{-9}$  mol) was calculated from the difference in the absorbance of free ruthenium(III) at 615 nm in a solution before and after immobilization. On the other hand, the amount of ruthenium(III)-immobilized film was estimated using LSV (Fig. S2, Supporting Information). The value ( $6.6 \times 10^{-10} \text{ mol/cm}^2$ ) was less than that ( $3.7 \times 10^{-8} \text{ mol/cm}^2$ ) obtained by spectrophotometry. Therefore, the total amount of the ruthenium(III) in the film was not related to the electrode reaction. The particle size of  $\beta$ -casein was reported at approximately 10 nm,<sup>33</sup> and the volume was estimated at  $5.23 \times 10^{-28} \text{ m}^3$ . For the formation of a casein-ruthenium(III) complex, the amounts of ruthenium(III) on the collagen film surface against the phosphate group of the casein may be 1:1, 2:1, and 3:1. Ruthenium(III) in the amount of  $4.0 \times 10^{-11}$  mol was combined with  $\alpha$ -casein of  $2.0 \times 10^{-9}$  M was combined with ruthenium(III) at 2:1. Because the amount

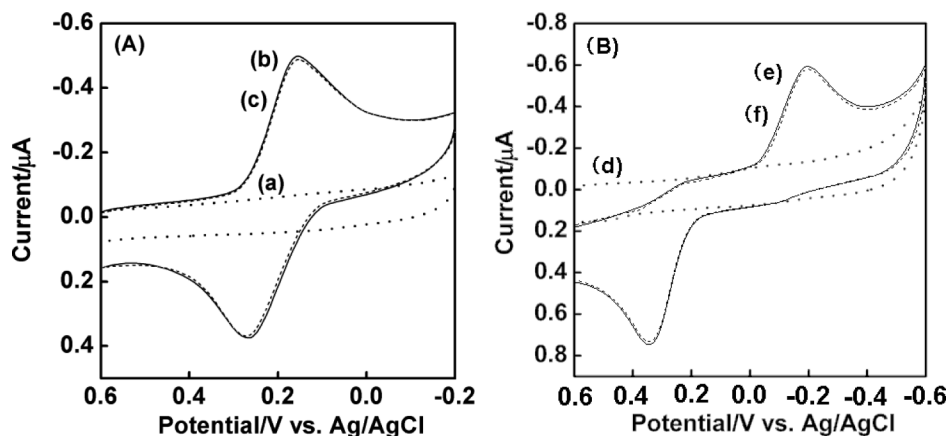


Fig. 6 Cyclic voltammograms of markers using a glassy carbon electrode. (A) (a) Blank; (b)  $3.0 \times 10^{-5}$  M  $[\text{Fe}(\text{CN})_6]^{3-}$ ; (c)  $3.0 \times 10^{-5}$  M  $[\text{Fe}(\text{CN})_6]^{3-} + 3.0 \times 10^{-8}$  M  $\alpha$ -casein. (B) Hydroquinone: (d) blank; (e)  $3.0 \times 10^{-5}$  M hydroquinone; (f)  $3.0 \times 10^{-5}$  M hydroquinone +  $3.0 \times 10^{-8}$  M  $\alpha$ -casein. The procedure for (c):  $3.0 \times 10^{-5}$  M  $[\text{Fe}(\text{CN})_6]^{3-} + 3.0 \times 10^{-8}$  M  $\alpha$ -casein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). The procedure for (d):  $3.0 \times 10^{-5}$  M hydroquinone +  $3.0 \times 10^{-8}$  M  $\alpha$ -casein were incubated for 10 min in 0.1 M acetate buffer (pH 3.2). Measurements were performed using cyclic voltammetry (scan rate,  $50 \text{ mV s}^{-1}$ ) after a potential of 0.2 V was applied to the electrode for 5 min with stirring.

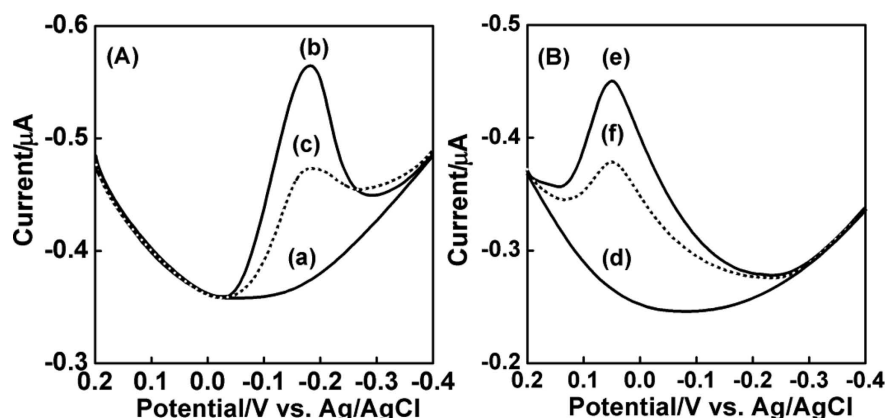


Fig. 7 Differential pulse voltammograms of  $\alpha$ -casein using a glassy carbon plate electrode. (A) Bare electrode: (a) blank; (b)  $2.5 \times 10^{-5}$  M ruthenium(III); (c)  $2.5 \times 10^{-5}$  M ruthenium(III) +  $5.0 \times 10^{-8}$  M  $\alpha$ -casein. (B) Ruthenium(III)-modified electrode: (d) without ruthenium(III); (e) with ruthenium(III); (f) with ruthenium(III) +  $1.0 \times 10^{-9}$  M  $\alpha$ -casein. The electrode was immersed for 10 min in 0.1 M phosphate buffer (pH 3.2) with  $\alpha$ -casein. measurements of  $\alpha$ -casein were performed using differential pulse voltammetry (scan rate,  $5 \text{ mV s}^{-1}$ ; pulse amplitude,  $50 \text{ mV}$ ; sample width,  $2 \text{ ms}$ ; pulse width,  $50 \text{ ms}$ ; and, pulse period,  $200 \text{ ms}$ ) after a potential of 0.2 V was applied to the electrode for 5 min with stirring.

of ruthenium(III) in the film was  $1.29 \times 10^{-10}$  mol, the complex formed sufficiently.

To confirm the immobilization of ruthenium(III) on the film, the reduction peak currents at scan rates ranging from  $25 - 200 \text{ mV s}^{-1}$  were measured using linear sweep voltammetry (Fig. S3, Supporting Information). Since the peak current of ruthenium(III) was proportional to the scan rate, the electrode response was a surface-controlled electrochemical process. The linear regression equation was expressed as follows: Peak current of ruthenium(III) without  $\alpha$ -casein ( $\mu\text{A}$ ) =  $-0.0401 + 0.00625v$  ( $\text{mV s}^{-1}$ ) ( $R = 0.997$ ). Furthermore, the peak current of ruthenium(III) with  $1.0 \times 10^{-9}$  M  $\alpha$ -casein increased in a linear fashion as the scan rate increased. Peak current ( $\mu\text{A}$ ) =  $-0.0111 + 0.00378v$  ( $\text{mV s}^{-1}$ ) ( $R = 0.995$ ). To examine whether

the peak current obtained by the ruthenium-immobilized electrode depended on the time with a potential, the time was changed in the range of from 1 and 7 min (Fig. S4, Supporting Information). The peak current of ruthenium(III) alone increased until 1.5 min and was constant for more than 2 min. The electrode response using a ruthenium(III)-film electrode with  $1.0 \times 10^{-9}$  M  $\alpha$ -casein decreased up to 4 min and plateaued for more than 4.5 min. On the other hand, voltammetric sensing of casein was performed using DPV because the reduction peak was too broad for the detection of casein.

Figure 7 shows the voltammograms of ruthenium(III) using a plain glassy carbon plate and the ruthenium(III)-modified plate in 0.1 M acetate buffer (pH 3.2). A reduction peak for  $2.5 \times 10^{-5}$  M ruthenium(III) was observed at  $-0.15 \text{ V}$ , and the peak

Table 2 Determination of casein in milk samples

| Sample    | Content of casein    |        |                       |
|-----------|----------------------|--------|-----------------------|
|           | Present method (g/g) | RSD, % | Reference value (g/g) |
| Dry milk  | 0.0467 ± 0.003       | 6.4    | 0.047                 |
| Skim milk | 0.262 ± 0.011        | 4.2    | 0.27                  |

Number of determinations ( $n = 5$ ).

1:50000 (dry milk) and 1:200000 (skim milk) dilutions with 0.1 M acetate buffer (pH 3.2).

current was decreased *via* the addition of  $5.0 \times 10^{-8}$  M  $\alpha$ -casein (Fig. 7(A)). The linear range of  $\alpha$ -casein obtained by a plain glassy carbon plate was  $5.0 \times 10^{-9}$  –  $6.0 \times 10^{-8}$  M (Fig. S5(A), Supporting Information). The peak current ( $\mu\text{A}$ ) =  $-0.0191 \times C_{\text{casein}}$  ( $10^{-8}$  M) + 0.195 ( $R = -0.998$ ). When the detection limit of  $\alpha$ -casein was estimated at 3-fold the standard deviation ( $3\sigma$ ), the detection limit for the bare electrode was  $3.0 \times 10^{-9}$  M. The RSD of  $5.0 \times 10^{-8}$  M  $\alpha$ -casein was 4.7% ( $n = 5$ ). The change in the electrode response was similar to that of  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  with  $\alpha$ -casein. In contrast, the voltammograms of  $\alpha$ -casein were also measured using a ruthenium(III) modified electrode with collagen film (Fig. 7(B)). A peak due to the formation of a ruthenium(III)-nitritotriacetic acid (NTA) appeared at 0.05 V. No peak with  $5.0 \times 10^{-8}$  M  $\alpha$ -casein was observed using the electrode coated with collagen film alone (not shown).

The calibration curve of  $\alpha$ -casein using a ruthenium(III)-modified electrode with collagen film was linear and ranged from  $7.5 \times 10^{-11}$  to  $2.0 \times 10^{-9}$  M (Fig. S5(B), Supporting Information). The peak current ( $\mu\text{A}$ ) =  $-0.0826 \times C_{\text{casein}}$  ( $10^{-9}$  M) + 0.212 ( $R = -0.996$ ). The RSD of  $1.0 \times 10^{-9}$  M  $\alpha$ -casein was 4.9% ( $n = 5$ ). The detection limit of  $\alpha$ -casein was  $5.0 \times 10^{-11}$  M, as estimated at 3-fold the standard deviation ( $3\sigma$ ). The sensitivity using the ruthenium(III)-modified electrode was improved by two orders of magnitude compared with that using a plain glassy carbon plate.

#### Detection of casein in milk using the sensor

The detection of casein was performed using an electrode modified with ruthenium(III)/collagen film. Samples of dried and skim milk were selected to confirm the power of the sensor (Table 2). The coexisting substances contained in each sample of the milk were as follows: dry milk (per 100 g) contained protein 11.7 g (amount of casein in total protein was 4.7 g), lipid 27.0 g, carbohydrate 56.3 g, sodium 140 mg, calcium 380 mg, and phosphorus 210 mg; and, skim milk (per 100 g) contained protein 34.0 g (amount of casein in total protein was 27 g), lipid 1.0 g, carbohydrate 53.3 g, sodium 410 mg, calcium 1200 mg, and phosphorus 960 mg. A sample (0.100 g) was dissolved in 0.1 M acetate buffer (pH 3.2), and dry milk and skim milk was diluted to 1:50000 and 1:200000 using the solution. The measurement of casein was performed in 0.1 M acetate buffer (pH 3.2) as a supporting electrolyte. The influence of calcium(II) was apparent in the voltammetric measurements of casein, as mentioned above. However, the amount of calcium(II) at this level had no influence in the measurement of casein.

The values obtained by the ruthenium(III)-modified electrode were in accordance with the values listed by the manufacturers. The relative standard deviations of dry milk and skim milk were inside 6.4 and 4.2% ( $n = 5$ ). Because the decrease in the electrode response depended mainly on the concentrations of

$\alpha$ - and  $\beta$ -caseins, the ruthenium(III)-modified electrode was applicable to the detection of casein in the milk. Watanabe *et al.* reported the results of a sandwich ELISA for the detection of casein using spectrophotometry. The detection limit of the method was  $3.3 \times 10^{-11}$  M.<sup>34</sup> In addition, an immunoassay of  $\beta$ -casein was attempted based on magnetic bead and gold nanoparticle probes.<sup>35</sup> The linear range of the assay was from  $2.8 \times 10^{-10}$  to  $6.4 \times 10^{-8}$  M. A chromatographic method for the separation and determination of caseins using hydrophobic interactions was also developed.<sup>36</sup> The sensitivity of this method was on a  $10^{-11}$  M level, which was similar to the methods mentioned above. The merits of using the electrode in the sensing of casein included low cost and simplicity.

## Conclusions

In the present study, a sensing of casein was achieved based on the interaction between its phosphate groups and ruthenium(III). When the binding occurred in a solution, the hydrophobicity of casein was drastically increased by its conformational change of casein. As a result, casein was detected using the decrease in the peak current due to the formation of the casein-ruthenium(III) complex. A ruthenium(III)-modified electrode sensor with collagen film was constructed. We carried out voltammetric measurements of casein in milk to examine the function of the sensor. The values obtained by this method agreed with the values certified by the manufacturers. Consequently, this sensor could provide a simple and rapid screening of casein as a food allergen.

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## Supporting Information

Figures S1 – S5 are in Supporting Information. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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