The Determination of a Small Amount of a Biological Constituent by Use of Chemiluminescence. XVI. High-Sensitive Detection of Proteins by an Ion-Exchange Chromatograph-Chemiluminescence Detector System

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(Received May 11, 1989)

An ion-exchange chromatograph-chemiluminescence detector system was developed by connecting a high-resolution ion-exchange column with a chemiluminescence detector using a 1,10-phenanthroline–hydrogen peroxide–copper(II) system which was first developed by the authors for the detection of protein. A sodium tetraborate–hydrochloric acid buffer solution was found to be suitable for the development of well-defined chromatogram without lowering the resolution of an ion-exchange column and the sensitivity of a chemiluminescence detector. The elution of protein was carried out by a gradient elution method. According to the present method, two mixture samples of ovalbumin–trypsin inhibitor (from soybean, crude) or bovine serum albumin–trypsin inhibitor (from soybean, crude)–trypsin inhibitor (from chicken egg white), which were too dilute to detect by a UV detector, gave chromatograms similar as in UV detection.

A flow-injection analysis (FIA) using the chemiluminescence (CL) detection was previously established by the authors[1] for the determination of a small amount of protein. The determination of protein is based on the lowering of the catalytic activity of a metal ion or a metal complex by the formation of a metal–protein complex in the presence of protein. The FIA using a 1,10-phenanthroline (phen)–hydrogen peroxide (H_2O_2)–copper(II) (Cu(II)) system as a CL system was about 10^2—10^6 times as sensitive as conventional methods such as colorimetry and fluorometry.[2-4] This method has been utilized as a detection method in immunoaffinity chromatography,[5] metal chelate affinity chromatography,[6] and chromatography using a zeolite column.[7] These applications made it feasible to selectively determine albumin in a serum sample or to separate and determine the individual constituent in a mixed albumin–globulin sample by employing isocratic elution or pH-gradient elution, respectively.

With the object of applying the CL detector to the separation and determination of several proteins in their mixtures, the present study deals with the connection of a CL detector and a high-resolution ion-exchange column. A buffer solution was first selected so as to give a well-defined chromatogram without lowering the resolution of an ion-exchange column and the sensitivity of the CL detector. The elution of proteins from the column was made by use of the salt solution in which the concentration of the salt was allowed to change continuously. Hereafter this elution is termed "salt-gradient elution". The method established on the basis of the experimental results was applied to two mixture samples of ovalbumin–trypsin inhibitor (from soybean, crude) (STI) or bovine serum albumin–STI–trypsin inhibitor (from chicken egg white) (TI), which were too dilute to detect by a UV detector. Chromatograms similar as in UV detection were obtained.

Experimental

Reagents. All reagents were of commercially available special grade. Deionized water was distilled for use.

A phen solution contained 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} ethylhexadecyldimethylammonium bromide, 2.0×10^{-7} mol dm^{-3} tetraethylenepentamine, and 1.0×10^{-1} mol dm^{-3} sodium hydroxide (NaOH) was prepared. A 6.0 wt% H_2O_2 solution was prepared by diluting 30 wt% H_2O_2 with water. A 1.0×10^{-7} mol dm^{-3} Cu(II) catalyst solution was prepared by diluting a 2.0×10^{-3} mol dm^{-3} Cu(II) stock solution (biuret reagent) with a buffer solution consisting of either 2.0×10^{-1} mol dm^{-3} boric acid (H_3BO_3) and 2.0×10^{-1} mol dm^{-3} potassium hydroxide (KOH) (Buff-A; pH 12.19) or 1.50×10^{-1} mol dm^{-3} sodium chloride (NaCl) and 3.96×10^{-1} mol dm^{-3} NaOH (Buff-B; pH 13.20).

The following buffer solutions were used for the equilibration of an ion-exchange column and for the feed of a protein solution: 5.0×10^{-3} mol dm^{-3} H_3BO_3–5.0×10^{-4} mol dm^{-3} KOH (Buff-C; pH 8.04), 9.5×10^{-3} mol dm^{-3} disodium hydrogenphosphate (Na_2HPO_4)–5.0×10^{-4} mol dm^{-3} sodium dihydrogenphosphate (NaH_2PO_4) (Buff-D; pH 8.23), 2.4×10^{-2} mol dm^{-3} Na_2HPO_4–1.0×10^{-3} mol dm^{-3} potassium dihydrogenphosphate (KH_2PO_4) (Buff-E; pH 8.33), 2.00×10^{-2} mol dm^{-3} Tris–1.17×10^{-2} mol dm^{-3} hydrochloric acid (HCl) (Buff-F; pH 8.00), and 2.0×10^{-2} mol dm^{-3} sodium tetraborate ($Na_2B_4O_7$)–1.7×10^{-4} mol dm^{-3} HCl (Buff-G; pH 8.04).

NaCl solutions obtained by dissolving NaCl in Buff-C—Buff-G were used as an eluent for salt-gradient elution. The following proteins were dissolved in Buff-C—Buff-G and diluted for use; ovalbumin (Ova) (Sigma Chemical Co.), STI (Sigma Chemical Co.), TI (Sigma Chemical Co.), human serum albumin (HSA) (Miles Laboratories, Inc.), bovine serum albumin (BSA) (Nakarai Chemicals, Ltd.), bovine serum γ-globulin (BγG) (Sigma Chemical Co.), human serum α_2-macroglobulin (α_2M) (Sigma Chemical Co.), apoferritin (ICN Pharmaceuticals, Inc.), and conalbumin (Sigma Chemical Co.).

Apparatus and Procedure. High-sensitive ion-exchange chromatography with CL detection was carried out using

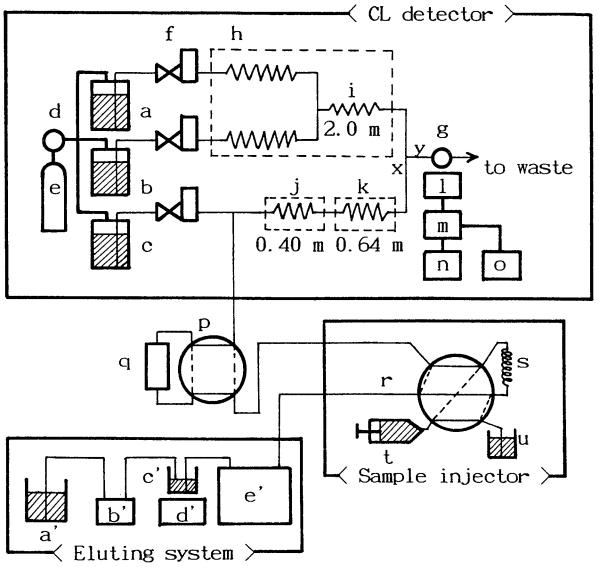


Fig. 1. Schematic diagram of the ion-exchange chromatograph–CL detector system. [CL detector] a: phen solution, b: H_2O_2 solution, c: Cu(II) catalyst solution, d: regulator, e: N_2 cylinder, f: flowmeter equipped with a needle valve, g: flow-cell, h: thermostat, i: mixing coil, j: reaction tube, k: cooling tube, l: photomultiplier, m: amplifier, n: recorder, o: integrator, p: four-way cock, q: column, [Sample injector] r: six-way cock, s: sampling loop, t: syringe, u: sample solution, [Eluting system] a′: NaCl solution, b′: peristaltic pump, c′: gradient-mixer, d′: magnetic stirrer, and e′: pump.

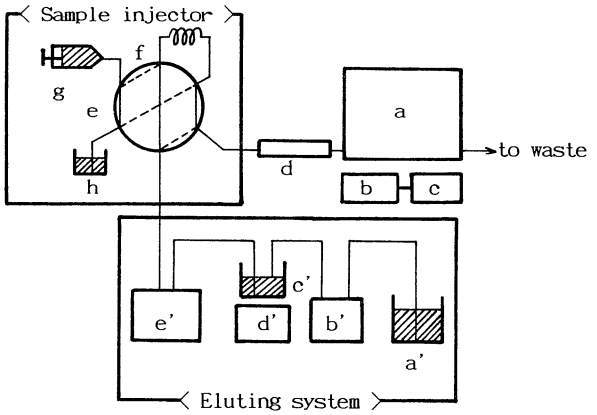


Fig. 2. Schematic diagram of the ion-exchange chromatograph–UV detector system. [UV detector] a: UV detector, b: recorder, c: integrator, d: column, [Sample injector] e: six-way cock, f: sampling loop, g: syringe, h: sample solution, [Eluting system] a′: NaCl solution, b′: peristaltic pump, c′: gradient-mixer, d′: magnetic stirrer, and e′: pump.

the high-performance liquid chromatography (HPLC) system (Fig. 1) equipped with a CL detector and an apparatus for the salt-gradient elution. The CL detector was operated as follows: All the tubes and connectors in the line were made of Teflon. Each solution of phen (a), H_2O_2 (b), and Cu(II) (c) was fed by means of 2.9×10^5 Pa gauge pressure of nitrogen gas; the flow rate was adjusted to 1.0 $cm^3\ min^{-1}$ by a flowmeter (f) (Gilmont) equipped with a needle valve. A phen solution and a H_2O_2 solution were mixed through a mixing coil (i) placed in a thermostat (h) held at 25 °C. After a carrier solution was fed at the flow rate 1.0 $cm^3\ min^{-1}$ by a pump (e′) (Nihon Seimitsu Kagaku Co., Ltd., NP-DX-3U). A 50 mm^3 sample of protein solution was introduced onto a 4.6 mm i.d. ×35 mm ion-exchange column (q) (Tosoh Co., TSKgel DEAE-NPR) by means of a sampling loop (s) and a six-way cock (r).

The protein held on the ion-exchange column was next subjected to the salt-gradient elution as follows: A definite concentration of NaCl solution (a′) was fed at a flow rate of 0.5 $cm^3\ min^{-1}$ by means of a peristaltic pump (b′) (PERISTA MINI PUMP SJ-1211) to the gradient-mixer (c′) in which 30 or 40 cm^3 of Buff-C—Buff-G had been previously placed. The solution in a gradient-mixer was well-stirred by a magnetic stirrer (d′) and was fed into ion-exchange column at a flow rate of 1.0 $cm^3\ min^{-1}$ using a constant flow pump. The protein eluted from the ion-exchange column was first mixed with a Cu(II) catalyst solution and then passed through a reaction tube (j) (0.40 m), held at 95 °C, and a cooling tube (k) (0.64 m), held at 0 °C, in that order. The reaction solution was further mixed with the phen–H_2O_2 solution, which had been throughly premixed by means of a 2.0 m mixing coil; the solution thus obtained was passed through a flow cell (g) (25 cm×0.8 mm i.d. poly(vinyl chloride) tube). The CL intensity of the solution passing through the cell was measured by means of a photomultiplier (g) (Hamamatsu TV Co., Ltd., R928), amplified with an amplifier (l) (Horiba Ltd., OPE-402), and recorded on both a recorder (m) (Yokogawa Electric Works, Ltd., 3046) and an integrator (n) (Shimadzu Co., Chromatopac C-R3A). The distance between x and y was 3 cm.

The FIA using direct-injection was carried out by injecting a 50 mm^3 portion of a protein sample solution into the eluent line and by operating a four-way cock and a six-way cock equipped with a sample loop.

The selection of a buffer solution was carried out using the HPLC system (Fig. 2) equipped with an ultraviolet spectrophotometer (UV) and an apparatus for the salt-gradient elution as follows: All the tubes and connectors in the line were Teflon-made. After a carrier solution (c′) was fed at the flow rate of 1.0 $cm^3\ min^{-1}$ by a pump (e′) (Nihon Seimitsu Kagaku Co., Ltd., NP-DX-3U), a 50 mm^3 of protein sample solution was introduced onto a 4.6 mm i.d.×35 mm ion-exchange column (d) (Tosoh Co., TSKgel DEAE-NPR) by means of a sample loop (f) and a six-way cock (e). The protein held on the ion-exchange column was next eluted by the salt-gradient elution as in high-sensitive ion-exchange chromatography with CL detection. The protein eluted from the ion-exchange column was detected at 280 nm by a UV detector (a) (Shimadzu Co., SPD-6a) and recorded by both a recorder (b) (Yokogawa Electric Works, Ltd., 3046) and an integrator (c) (Shimadzu Co., Chromatopac C-R3A).

Results and Discussion

Development of Various Chromatography Columns–CL Detector Systems. No CL detector has been used for the post-column detection of protein, such a

detector is highly sensitive but it is apt to be influence by the measurement conditions. However, the authors have succeeded in the following separation and determination of protein using the combinations between each column and a CL detector by the optimization of the measurement conditions: 1) Separation and determination of 3×10^{-4}—1×10^{-1} g dm^{-3} HSA by the isocratic elution with an HCl–potassium chloride (KCl) buffer solution (pH 2.2) on the immunoaffinity column–CL detector system.[5] 2) Separation and determination of BSA BγG, and lysozyme in each 1.0×10^{-4}—1.0×10^{-1} g dm^{-3} by the pH-gradient elution (pH 8.0→4.0) with an aqueous acetic acid solution on the metal chelate affinity column–CL detector system.[6] 3) Separation and determination of HSA in 1×10^{-5}—5×10^{-3} g dm^{-3} and of HγG in 2×10^{-5}—5×10^{-3} g dm^{-3} by the isocratic elution with a NaOH–NaCl buffer solution (pH 12.0) on the zeolite column–CL detector system.[7]

In the present study, CL detection has been combined for the first time with ion-exchange chromatography in which an eluent was unnecessary for any organic solvent. Separation of proteins of more than two kinds was feasible. Salt-gradient elution was first used since both salt-gradient elution and ion-exchange chromatography had not previously been examined by the authors for the separation and determination of proteins.

Salt-Gradient Elution. In ion-exchange chromatography, it is rare to elute the desired substances with a starting buffer solution alone, because it takes too much time and allows the concentration of the desired substances to diminish. In those circumstances the elution method, in which the ability for eluting the desired substance is gradually enhanced, is often used. To enhance the eluting ability, the following two procedures are supposed: 1) an increase in the ionic strength of an eluent, and 2) a change in pH of an eluent. Since it is difficult to change the pH of an eluent linearily, the latter is seldom used. The former elution procedure which depends upon increasing the ionic strength by the addition of a salt at a definite pH is usually employed. Two methods for changing the ionic strength of an eluent are as follows: One is the stepwise method and another is the continuous method (gradient method). In the present study, the salt-gradient elution, in which the ionic strength of an eluent was changed continuously and linearily, was adopted by the eluting system shown in Figs. 1 and 2. The following four salts were examined for the salt-gradient elution: NaCl and KCl consisting of a monovalent cation and a monovalent anion, magnesium chloride ($MgCl_2$) consisting of a bivalent cation and two monovalent anions, and sodium sulfate (Na_2SO_4) consisting of two monovalent cations and a bivalent anion. Neither $MgCl_2$ nor Na_2SO_4 gave well-defined chromatograms, but NaCl and KCl gave an identical and well-defined chromatogram. In the present study, inexpensive NaCl was used exclusively. The concentration change of a salt in an eluent was derived from the balance equation in a continuous stirred tank reactor (CSTR) as follows:

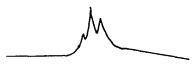
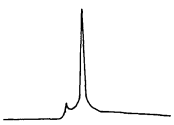
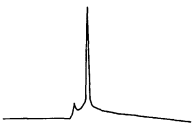
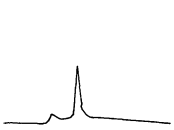
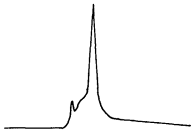
$$C_A = C_{A,0} \times (v_2 - v_1) \times t/V_0 \quad (1)$$

where t (min): gradient time, C_A (mol dm^{-3}): concentration in a gradient-mixer (c′) after t minutes, $C_{A,0}$ (mol dm^{-3}): concentration of NaCl (a′) fed to a gradient-mixer (c′), v_1 (=0.5 cm^3 min^{-1}): flow rate of NaCl (a′) fed to a gradient-mixer (c′), v_2 (=1.0 cm^3 min^{-1}): flow rate of an eluent (c′) fed to an ion-exchange column, and V_0 (cm^3): initial volume in a gradient-mixer (c′) at t=0.

Equation 1 was valid only when $v_2=2v_1$. Therefore C_A was proportional to t when $v_2=2v_1$. This was confirmed by the argentometric titration of chloride in an eluent.

Selection of an Optimum Buffer Solution. The buffer solutions as carrier solutions of the protein were selected on the following basis. H_3BO_3–KOH Buffer solution (Buff-C),[1,6,8–11] Na_2HPO_4–NaH_2PO_4 buffer solution (Buff-D),[7] and Na_2HPO_4–KH_2PO_4 buffer solution (Buff-E)[5] were used successfully as carrier solutions of the protein in FIA and HPLC with a CL detector which were reported by the authors. Tris–HCl buffer solution (Buff-F) was widely used in the

Table 1. Selection of an Optimum Buffer Solution

Buffer solution	Response[a] without protein	Response[a] with protein
Buff-C		
Buff-D		
Buff-E		
Buff-F		
Buff-G		

a) Each response shows an absorbance (280 nm) curve against a 10-min linear salt-gradient (0→0.5 mol dm^{-3} NaCl).

column charged with weakly basic anion-exchange resin.[12-14] Since Buff-C—Buff-F were not suitable for the present purpose, as shown from Table 1 and Fig. 3, $Na_2B_4O_7$-HCl buffer solution (Buff-G) was finally selected so as to satisfy the following two conditions: 1) The buffer solution was around pH 8 which was generally used in anion-exchange chromatography of proteins, and 2) the buffer solution did not give any slightly soluble copper(II) precipitate or stable copper-(II) complex compound. To optimize the buffer solution as a carrier solution, the responses recorded using the apparatus shown in Fig. 2 (UV) and Fig. 1 (CL) were examined. The results in Table 1 show the respeonses obtained applying salt-gradient elution with or without injecting 1.0×10^{-1} g dm^{-3} BγG after the column in Fig. 2 was equilibrated with various buffer solutions. As can be seen from Table 1, Buff-C, Buff-D, and Buff-E could not be used for the acquisition of a correct response for protein, but Buff-F and Buff-G gave the possibility for it.

The applicability of Buff-F and Buff-G to a CL system was next examined using the FIA apparatus shown in Fig. 1. The results obtained are shown in Fig. 3. Though in case of Buff-F the CL intensity of the base-line was small and distinct responses for BγG were not observed, in case of Buff-G the CL intensity of the base-line was large and distinct responses for BγG were observed. As can be seen from Table 2, the result in case of Bufff-F was supposed to be due to the complex formation between Cu(II) and Tris as a component of Buff-F. Accordingly Buff-G was used as a carrier solution in the following experiment pertaining to the separation and CL detection of proteins.

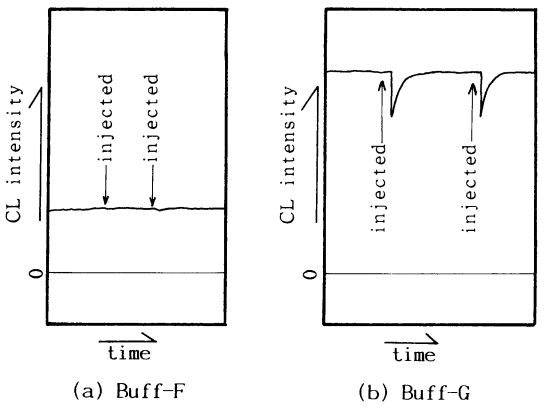


Fig. 3. Comparison of Buff-F and Buff-G in a CL system. Sample: 50 mm^3 of 1.0×10^{-3} g dm^{-3} BγG.

Table 2. Formation Constant of Cu(II) Complex

Ligand	Formation constant	
Tris	log K_1 3.98 log K_2 3.49 log K_3 3.20	log K 10.67
EDTA	log K_1 18.79	log K 18.79
NH_3	log K_1 4.27 log K_2 3.55 log K_3 2.90 log K_4 2.18	log K 12.90

Selection of an Optimum Buffer Solution for a Cu(II) Catalyst Solution. A mixture sample consisting of 1.0×10^{-3} g dm^{-3} Ova and 1.0×10^{-3} g dm^{-3} STI as model proteins was separated by using an apparatus in Fig. 1 (CL) and a Cu(II) catalyst solution (c) containing Buff-A (Fig. 4). Here, a linear salt-gradient elution was applied for 10 minutes in the range of

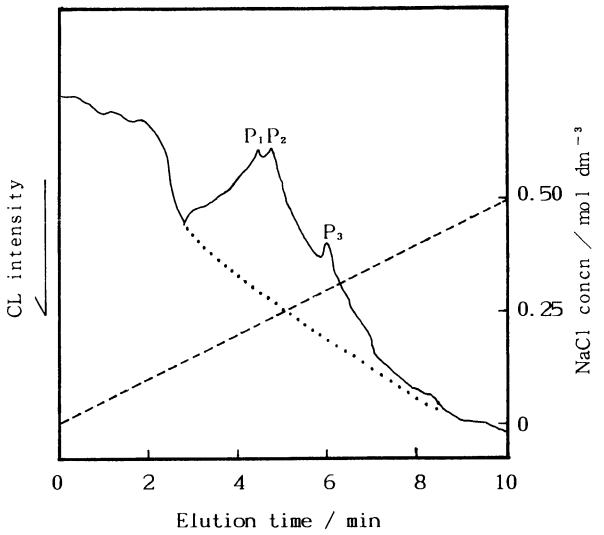


Fig. 4. Chromatograms for a protein mixture in case of Buff-A. Sample: 50 mm^3 of 1.0×10^{-3} g dm^{-3} Ova and 1.0×10^{-3} g dm^{-3} STI.

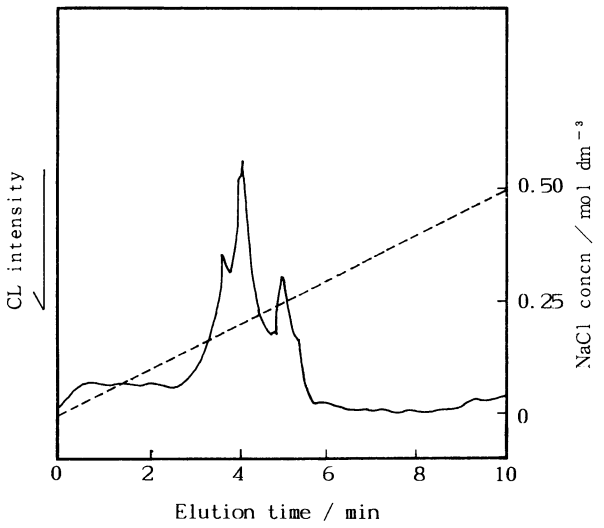


Fig. 5. Chromatograms for a protein mixture in case of Buff-B. Sample: 50 mm^3 of 1.0×10^{-3} g dm^{-3} Ova and 1.0×10^{-3} g dm^{-3} STI.

0→0.5 mol dm^{-3} NaCl. P_1, P_2, and P_3 in Fig. 4 showed the responses for proteins. The dotted line showed a base-line which was confirmed by a blank experiment. As can be seen from Fig. 4, the dotted line (base-line) varied markedly with the elution time. This was attributed to an increase of NaCl concentration with elution time in a CL system.

To eliminate the unfavorable effect of NaCl concentration on a base-line, Buff-B was used instead of Buff-A in Fig. 1. The pH at the reaction (j) between Cu(II) and protein was adjusted to about 13, and that at flow cell (g) to about 10.5, as optimized in the previous paper.[7] The result was shown in Fig. 5. As can be seen from Fig. 5, a satisfactory result almost was obtained with a 10-min linear gradient from 0 to 0.5 mol dm^{-3} NaCl. Though a buffer solution containing higher concentration of NaCl was further examined, no improvement could be obtained for the stabilization of the base-line.

Effect of Gradient Time. The effect of gradient time on the chromatogram was examined for the separation of a mixture sample consisting of 1.0×10^{-3} g dm^{-3} Ova and 1.0×10^{-3} g dm^{-3} STI as model proteins (Fig. 6). Here, linear salt-gradient elution was applied for 10—50 minutes in the range of 0→0.5 mol dm^{-3} NaCl. The results showed that separation of proteins was generally improved, but their peaks became much smaller with an increase of elution time.

The chromatogram (CL) obtained with a 30-min linear gradient from 0 to 0.5 mol dm^{-3} NaCl is shown in Fig. 7 together with that (UV) obtained with a 10-min linear gradient from 0 to 0.5 mol dm^{-3} NaCl. As can be seen from Fig. 7, both chromatograms were roughly similar in their shapes, and a CL detector was much more sensitive than a UV detector. Separation and detection of a mixed sample consisting of 1.0×10^{-3} g dm^{-3} Ova and 1.0×10^{-3} g dm^{-3} STI were difficult using a UV detector, but those of a mixed sample consisting of 1.0×10^{-4} g dm^{-3} Ova and 1.0×10^{-4} g dm^{-3} STI were feasible using a CL detector.

Separation of a Mixture Sample. The retention times of individual proteins were examined with a 10-min linear gradient from 0 to 0.5 mol dm^{-3} NaCl before the present method was applied to a mixture sample (Table 3). Since the retention times of STI, TI, and BSA were far apart from each other, these proteins

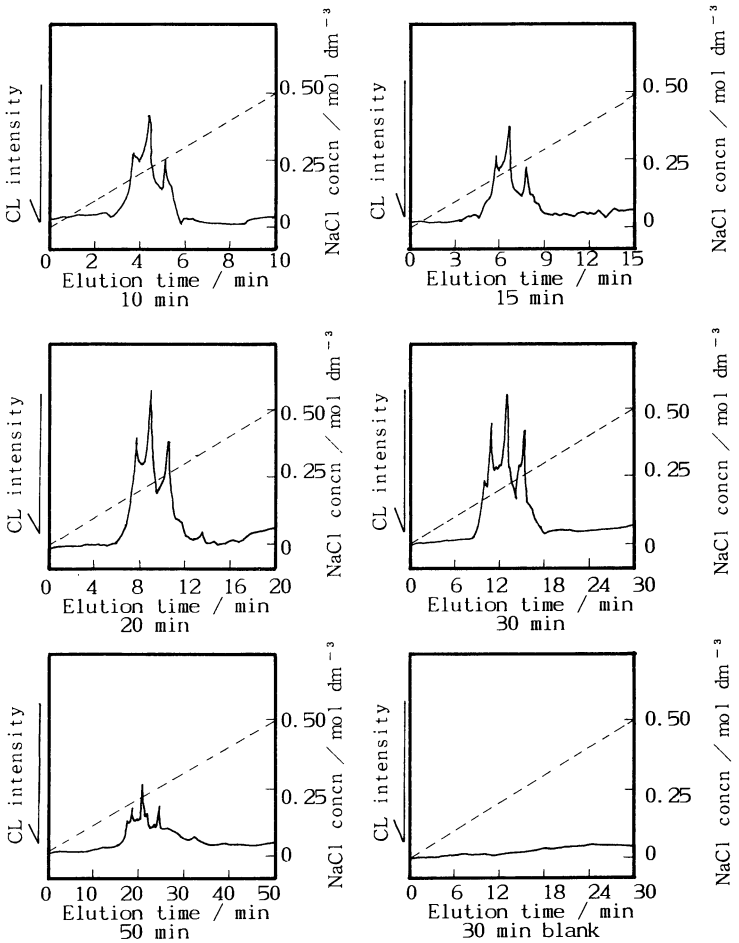
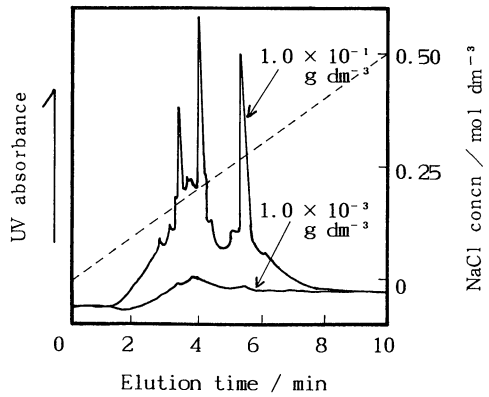
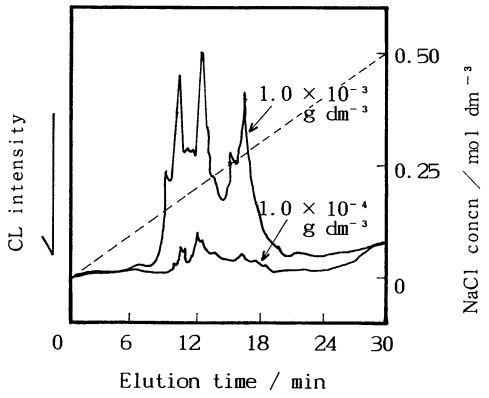


Fig. 6. Relationship between elution time and chromatogram. Sample: 50 mm^3 of 1.0×10^{-3} g dm^{-3} Ova and 1.0×10^{-3} g dm^{-3} STI.



(a) Chromatogram using a UV detector.



(b) Chromatogram using a CL detector.

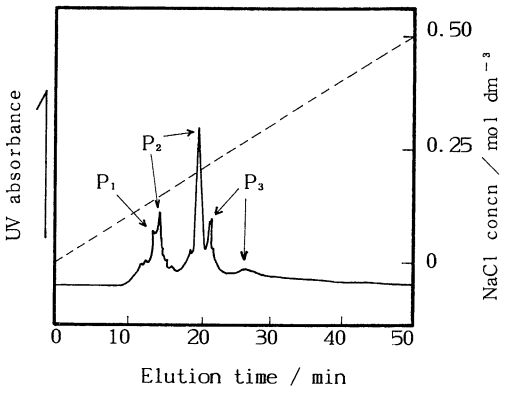
Fig. 7. Comparison of both detectors for the separation of a model sample. Sample: Ova and STI.

Table 3. Retention Times of Individual Proteins

Protein	Retention time/min
Ova	2.93, *3.40
STI	3.57, *4.97
TI	*2.96
HSA	*5.41
BSA	*5.34, 6.40
BγG	*3.80
α_2M	*5.59
Apoferritin	*7.03
Conalbumin	*2.33

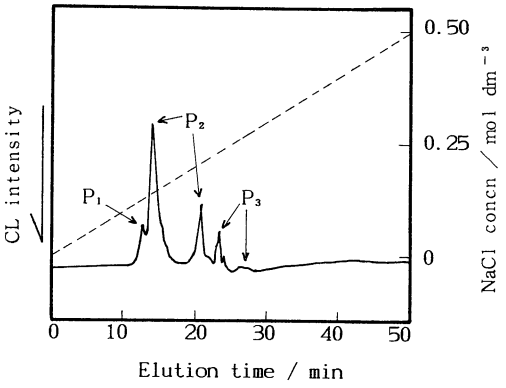
* means the retention time of the principal peak.

were used for the preparation of a mixed sample and the sample so obtained was subjected to separation. The chromatograms obtained with a 50-min linear gradient from 0 to 0.5 mol dm^{-3} NaCl using a CL detector and a UV detector were shown in Fig. 8. As can be seen from Fig. 8, the chromatogram obtained by connecting a CL detector with an ion-exchange column was almost satisfactory even with a hundredth of the concentration required for a UV detector.



(a) Chromatogram using a UV detector.

Sample: 50 mm^3 of 1.0 × 10^{-1} g dm^{-3} TI(P_1), 1.0 × 10^{-1} g dm^{-3} STI(P_2), and 1.0 × 10^{-1} g dm^{-3} BSA(P_3).



(b) Chromatogram using a CL detector.

Sample: 50 mm^3 of 1.0 × 10^{-3} g dm^{-3} TI(P_1), 1.0 × 10^{-3} g dm^{-3} STI(P_2), and 1.0 × 10^{-3} g dm^{-3} BSA(P_3).

Fig. 8. Ion-exchange chromatograms of a mixture sample.

The High-Sensitive Detection of Proteins by an Ion-Exchange Chromatograph-CL Detector System. The high-sensitive ion-exchange chromatograph–CL detector system for the detection of a small amount of protein was established by connecting a CL detector with a high-resolution ion-exchange column. Its connection was made feasible by introducing a $Na_2B_4O_7$–HCl buffer solution (Buff-G; pH 8.04) by which a well-defined chromatogram of a protein could be obtained without lowering the resolution of the ion-exchange column and the sensitivity of the CL detector, and by introducing a salt-gradient elution which had not previously been examined for the separation and determination of protein by the authors. By injecting 50 mm^3 of mixture sample containing each 1.0×10^{-4} g dm^{-3} protein (pI=3.9—6.8) into a column and by applying salt-gradient elution (0→0.5 mol dm^{-3} NaCl) to the sample for 30 or 50 min, each protein could be separated and identified.

A CL detector gave a paek even when the protein was too dilute to be detected by a UV detector. It also gave a peak corresponding to the peak given by a UV detector. The chromatograms obtained by both detectors did not coincide since they were based on quite different principles. The results suggest that any proteins which can be separated by an ion-exchange column may be sensitively detected by the present method.

The present study was supported by a Grant-in-Aid for Scientific Research No. 63550567 from the Ministry of Education, Science and Culture.

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