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# CLEAVAGE OF HUMAN SERUM IMMUNOGLOBULIN G BY AN IMMOBILIZED PEPSIN PREPARATION

TSUGIKAZU TOMONO, TOHRU SUZUKI and EIICHI TOKUNAGA

*Plasma Fractionation Department, The Japanese Red Cross Central Blood Center 1-31, Hiroo 4, Shibuya-ku, Tokyo 150 (Japan)*

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**In order to obtain an efficacious and safe immunoglobulin G (IgG) preparation for intravenous use, the digestion of IgG with an immobilized pepsin (EC 3.4.23.1) preparation was studied. Thus, pepsin was immobilized onto glutaraldehyde-activated AH-Sepharose 4B under acidic conditions. The enzymatic properties, such as proteolytic activity, pH-activity profile and heat stability, of the immobilized pepsin preparation were examined. The immobilized pepsin retained more than 40% of its proteolytic activity toward *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine and more than 30% toward IgG, and also remarkable stability as compared with free pepsin. The immobilized pepsin thus prepared was efficiently used for the limited cleavage of IgG and the gel-filtration effect of the column made it easily possible to yield the $F(ab')_2$-rich fraction for intravenous use.**

## Introduction

The limited cleavage of human immunoglobulin G (IgG) with pepsin (EC 3.4.23.1) has been studied in order to obtain the $F(ab')_2$ fragment, which can be intravenously administered for passive immunotherapy with less side-effects as compared with a commercial IgG. The use of an immobilized enzyme preparation to obtain the $F(ab')_2$ fragment offers some advantages, that is, the prevention of contamination with pepsin in the final product, or the repeated use of pepsin.

One of the most helpful procedures for immobilization of enzymes is the CNBr method involving the binding of enzyme onto a matrix at alkaline pH. However, since pepsin is rapidly and completely inactivated in the pH region higher than 6.0, its attachment to a matrix must be carried out at acidic pH. Therefore, in order to obtain insoluble pepsin derivatives with high proteolytic activity, many investigators have used several methods. They included the fixation of pepsin either to a polydiazonium salt of polyaminostyrene [1,2], to a nitrated copolymer of methacrylic acid and methacrylic acid *m*-fluoroanilide [3,4], to an amino derivative of Sepharose by Ugi reaction [5], to an aminosilane derivative of porous glass with water-soluble carbodiimide [6,7], or to an amino or carboxyl derivative of 2-hydroxyethylmethacrylate gel with water-soluble carbodiimide [8]. Ryle [9] reported, in addition, that the pepsin immobilized on glutaraldehyde-activated aminoethyl-cellulose (AE-cellulose) had higher proterolytic activity than any other immobilized pepsin preparations mentioned above. However, AE-cellulose has low mechanical strength as a matrix.

In the present study, therefore, pepsin was immobilized onto glutaraldehyde-activated AH-Sepharose in order to improve the mechanical strength of the matrix obtained and to gain operational advantages. The immobilized pepsin thus obtained was characterized in detail and its application to the limited cleavage of IgG was studied.

Abbreviations: IgG, immunoglobulin G; AcPhe($I_2$Tyr), *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine; HPLC, high-performance liquid chromatography.

## Materials and Methods

*Materials* Porcine pepsin (twice crystallized, Grade 1 60 000 from Sigma Chem. Co ) was used without further purification AH-Sepharose 4B containing 8 μmol aminohexyl groups/ml swollen gel was purchased from Pharmacia Fine Chem Co *N*-Acetyl-L-phenylalanyl-L-3,5-diiodotyrosine (AcPhe($I_2$Tyr)), used for the assay of peptic activity, was purchased from Sigma Human IgG was industrially prepared from pooled human plasma by the method of Cohn et al [10] in the Japanese Red Cross Central Blood Center Glutaraldehyde (25% aqueous solution) and the other reagents of analytical reagent quality were obtained from Wako Pure Chem Ind , Ltd The concentrations of proteins used were spectrophotometrically determined using values of 13 8 (IgG) [11] and 14 9 (Pepsin) [12] as $E^{1\%}_{280nm}$ The molecular weight of pepsin was estimated at 35 400 [13]

*Preparation of immobilized pepsin* The AH-Sepharose was first washed with 0 5 M NaCl and then with water to remove remaining NaCl The washed AH-Sepharose was suspended in 5 vol 0 1 M phosphate buffer (pH 6 90) To the suspension was added glutaraldehyde (25% aqueous solution) in the ratio of 0 32 ml/ml AH-Sepharose gel (the mol ratio of glutaraldehyde to aminohexyl groups was 100 1), and the mixture was stirred at room temperature for 4 h to couple all amino groups to aldehyde groups The glutaraldehyde-activated matrix washed with the same buffer was then resuspended in 5 vol 0 1 M phosphate buffer (pH 5 90) and the pepsin solution was added to this The amount of pepsin added ranged from 5 to 100 mg/ml glutaraldehyde-activated gel The reaction was carried out for 20 h at 5°C with continued stirring The mixture was filtered and washed with the same buffer to remove the unreacted pepsin Then, 0 5 M ethanol of pH 5 90, adjusted with dilute HCl, was added and allowed to stand for 2 h at room temperature in order to inactivate the remaining aldehyde groups Finally, the immobilized pepsin gel thus prepared was alternately washed three times with 0 1 M acetate buffer (pH 4 0)/0 5 M NaCl and with 0 01 M HCl

*Determination of amount of pepsin bound on the matrix* 50 mg dried immobilized pepsin gel were suspended in 5 ml of 6 M HCl The suspension was then kept at 110°C for 24 h yielding dark-colored hydrolysates The acid was removed by the flash evaporator The residue was dissolved in the appropriate amount of 0 2 M citrate buffer (pH 2 2) and analyzed on a Hitachi liquid chromatograph amino acid analyzer Model 034 The amount of pepsin bound on the matrix was estimated from the contents of eight amino acids, that is, Asx, Glx, Gly, Ala, Val, Ile, Leu and Phe, in the matrix as compared with the contents of those amino acids in pepsin [13]

*Assay of peptic activity using AcPhe($I_2$Tyr) as substrate* The assay of proteolytic activities of both free and immobilized pepsin using AcPhe($I_2$Tyr) as substrate was carried out according to the method described by Jackson et al [14], involving the determination of the amount of L-3,5-diidodtyrosine released from AcPhe($I_2$Tyr) at 37°C within 10 min by ninhydrin color-reaction

A substrate solution was prepared by dissolving 99 4 mg AcPhe($I_2$Tyr) in 10 ml 0 1 M NaOH, followed by diluting to a proper concentration with distilled water and adjusting to a proper pH value with dilute HCl To prepare ninhydrin solution, 20 g ninhydrin and 0 38 g stannous chloride were dissolved in 750 ml ethyleneglycol monomethyl ether and to this 250 ml 4 M acetate buffer (pH 5 5) was added

To 5 ml of the AcPhe($I_2$Tyr) solution of appropriate concentration 0 5 ml pepsin solution was added or immobilized pepsin suspension which contained the corresponding amount of pepsin, and the mixture incubated at 37°C After 10 min, 2 5 ml ninhydrin solution were added to the reaction vessel and then it was placed in a boiling water for 15 min After cooling the mixture, 5 ml 50% ethanol were added, and it was subjected to colorimetry at 578 nm using a Cary 219 spectrophotometer The absolute hydrolysis rate (mM/min) of AcPhe($I_2$Tyr) was calculated from a standard absorbance curve made with L-3,5-diiodotyrosine The specific activities ($min^{-1}$) of both free and immobilized pepsin were calculated as the hydrolysis rates of AcPhe($I_2$Tyr) per molar solution of pepsin

*Assay of peptic activity using IgG as substrate* In order to assay the proteolytic activity of both free and immobilized pepsin using IgG as substrate, a pH-stat instrument Model PS-11 (Hiranuma Sangyo Co , Ltd ) was used

9 ml 0 1 M potassium chloride solution containing IgG, which was adjusted to pH 4 05 by adding dilute HCl, were placed in the titration vessel It was allowed to stand for 5 min at 37°C The end point was set at pH 4 0 and the titrator started A small amount of acid uptake of 0 02 M HCl was observed and then a flat base line was recorded 1 ml pepsin solution or immobilized pepsin suspension was added into the vessel and acid uptake was recorded for 15 min The final concentration of IgG was 20 mg/ml, and pepsin was about 1 wt % of IgG The absolute hydrolysis rates (mM/min) of peptide bonds were calculated by assuming that the apparent $pK_a$ of α-carboxyl and α-amino groups produced by the cleavage of peptide bond were 4 0 and 8 0, respectively The specific activity ($min^{-1}$) was calculated as the absolute hydrolysis rate of IgG per molar solution of pepsin

*High-performance liquid chromatography (HPLC)* The aqueous gel permeation chromatographic analysis of peptic digestion of IgG was achieved using a high-performance liquid chromatograph Model HLC-802 (Toyo Soda Manufacturing Co , Ltd ), equipped with two TSK-G3000SW columns in tandem Sample volume was 100 μl Elution buffer was 0 05 M sodium acetate (pH 5 0)/0 1 M sodium sulfate Flow rate was 1 ml/min Proteins eluted were detected spectrophotometrically at 280 nm

*Peptic cleavage of IgG using batch method* Digestions of IgG with free and immobilized pepsin were carried out at 37°C in 0 1 M acetate buffer (pH 4 0)/ 0 15 M NaCl Digests were adjusted to pH 8 5 by adding 0 1 M Tris buffer (pH 8 5) and 1 M NaOH in order to inactivate pepsin

*Limited cleavage of IgG through an immobilized pepsin column* The immobilized pepsin gel (15 ml) was placed into a glass column (10 mm diameter) The column was equilibrated with 0 1 M acetate buffer (pH 4 0)/0 15 M NaCl, and then into this column the 20 mg/ml of IgG solution was continually introduced at a flow rate of 6 5 ml/h at 37°C The eluate was analyzed by HPLC

## Results and Discussion

*Preparation of immobilized pepsin* The treatment of AH-Sepharose with glutaraldehyde resulted in coloring to brownish yellow of the matrix and gave an absorption band at 1 720 $cm^{-1}$ in the infrared absorption spectrum, due to the carbonyl groups This indicated the introduction of aldehyde groups onto the matrix Fig 1 shows the relationship between the amount of pepsin added to glutaraldehyde-activated matrix and the amount of pepsin bound on the matrix The addition of pepsin up to 50 mg/ml matrix resulted in increase of pepsin bound The maximum amount of pepsin bound was about 4 mg/ml

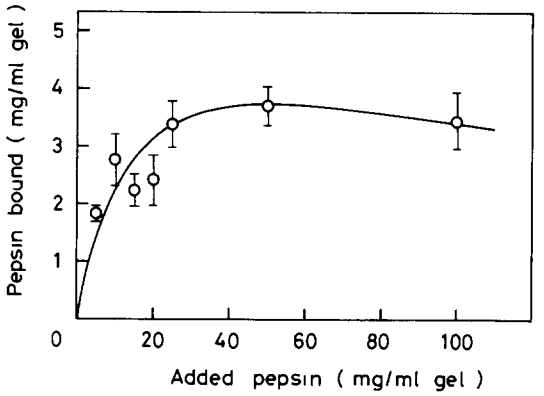


Fig 1 Immobilization of pepsin onto the glutaraldehyde-activated matrix The amount of pepsin fixed was determined by amino acid analysis of the immobilized pepsin gel

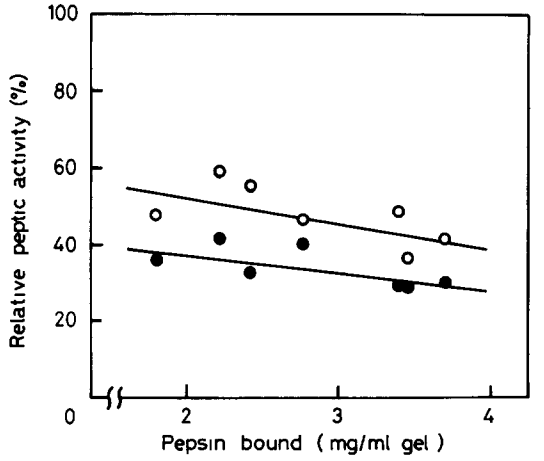


Fig 2 Effect of the amount of pepsin bound to the matrix on the proteolytic activities The relative peptic activity was the ratio of specific activity of immobilized pepsin to that of free pepsin The specific activities toward 0 145 mM AcPhe($I_2$Tyr) at pH 2 0 and 37°C (○), or toward 20 mg/ml IgG at pH 4 0 and 37°C (●) were determined as described in the experimental section

matrix, which indicated that only about 1 mol percent of all aminohexyl groups was used in the binding of pepsin molecules The result suggested that the modification of AH-Sepharose with glutaraldehyde sterically prevented the penetration of pepsin molecules into the glutaraldehyde-activated matrix, because glutaraldehyde polymerized itself [15] and covered the matrix surface

*Effect of amount of immobilized pepsin on the peptic activity* Fig 2 shows the effects of amount of immobilized pepsin on the relative peptic activities toward AcPhe($I_2$Tyr) and IgG The relative activity of immobilized pepsin was from 40 to 60% for AcPhe($I_2$Tyr), and from 30 to 40% for IgG The relatively high peptic activities of immobilized pepsin preparations might be due to the fact that pepsin molecules had only two aldehyde-reactive sites, i e , an $\epsilon$-amino group of lysine and an $\alpha$-amino group of N-terminal, that were located far from the proteolytic active center of the molecule That is, pepsin was immobilized on the matrix so that it could retain its native conformation to some extent

Fig 2 also shows that the relative peptic activities of immobilized pepsins decreased with the increase of pepsin contents in both cases These results suggested that pepsin molecules interfered with each other in the proteolytic reactions of AcPhe($I_2$Tyr) and IgG with the increase of pepsin contents on the matrices

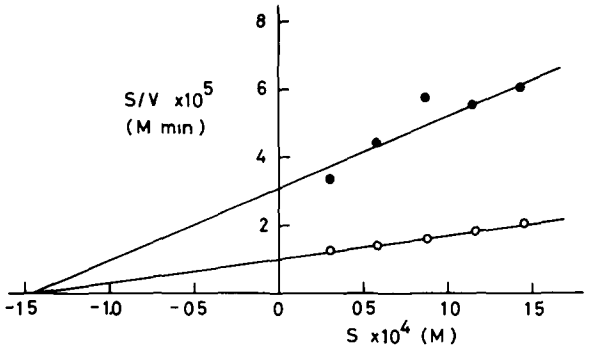


Fig 3 Hanes-Woolf plots for free and immobilized pepsin The hydrolysis of AcPhe($I_2$Tyr) with 0 36 $\mu$M free pepsin (○) and 0 66 $\mu$M immobilized pepsin fixed at the ratio of 3 39 mg/ml gel (●) were carried out at pH 2 0 and 37°C as described in the experimental section Hanes-Woolf equation $S/V = K_m/k_0 + S/k_0$, where $S$ is AcPhe($I_2$Tyr) concentration (M), $V$, hydrolysis rate of AcPhe($I_2$Tyr) per molar solution of pepsin ($min^{-1}$), $K_m$ Michaelis constant (M), $k_0$, molecular activity ($min^{-1}$)

*Kinetic parameters of hydrolysis of AcPhe($I_2$Tyr)* In order to characterize the immobilized pepsin, the kinetic parameters of the peptic hydrolysis of AcPhe($I_2$Tyr) were determined according to the Hanes-Woolf plots as shown in Fig 3 The Michaelis constant, $K_m$, for the immobilized pepsin was identical with that for free pepsin, 1 45 $10^{-4}$ M, while the molecular activities, $k_0$, for free and immobilized pepsin were 14 3 and 4 85 $min^{-1}$, respectively These results indicated that the lower proteolytic activity of immobilized pepsin was mainly due to the low molecular activity of pepsin molecules fixed, but not due to the restriction of diffusion of substrate This might be explained by the fact that pepsin molecules were fixed mainly on the surface of the matrix gel, as previously described in Fig 1 That is, the penetration of substrate into the matrix was not an important rate-determining factor in the proteolytic reaction The molecular activity of each pepsin molecule fixed was more important as an activity-controlling factor as compared with its Michaelis constant, which was under the influence of diffusion of the substrate Therefore, the effect of the amount of pepsin on the proteolytic activity, as shown in Fig 2, was presumably due to the differences of molecular

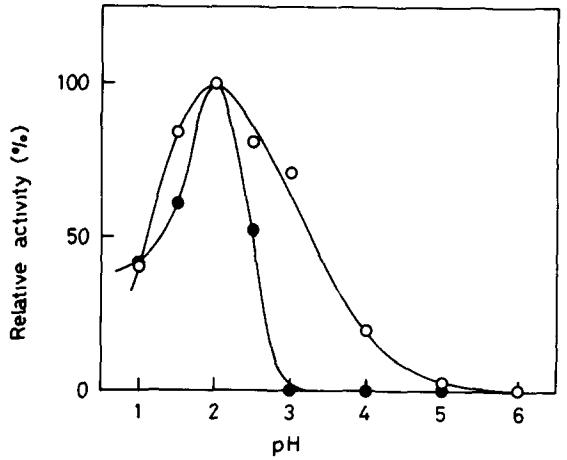


Fig 4 The pH dependence of peptic activity for free and immobilized pepsin The hydrolysis of AcPhe($I_2$Tyr) (0 145 mM) with 0 45 $\mu$M free pepsin (○) and 0 99 $\mu$M immobilized pepsin fixed on the matrix in the ratio of 3 70 mg/ml gel (●) were carried out at the appropriate pH and 37°C The specific activities were plotted as the percentage of the activity at pH 2 0, which was set at 100%

activities of pepsin molecules fixed, involving the conformational differences of pepsin molecules on the matrix

*Effect of pH on the peptic activity* Fig 4 shows the relationships between pH and the peptic activities of both free and immobilized pepsin toward AcPhe($I_2$Tyr) The pH-activity curve of immobilized pepsin was constricted without any significant displacement of the pH optimum (2 0) as compared with that of free pepsin Similar effects also observed in the case of pepsin fixed on the amino-derivative of a hydroxyalkyl methacrylate gel with water-soluble carbodiimide [8], or β-fructofuranosidase fixed on a diazonium salt of polyaminostyrene [16]

Although immobilization with glutaraldehyde has frequently resulted in an acid shift of optimal pH, as observed in the typical case of glucoamylase [17], or aldolase [18], because of the cationic properties of the glutaraldehyde-treated matrix, the same was not true in the present experiment This result was presumably due to the fact that the optimal pH of pepsin was in a very acidic pH region, where the proton concentration of water molecule itself as a solvent was so high that the proton concentration was not remarkably influenced by the presence of the matrix

*Stability of immobilized pepsin* Fig 5 shows the time dependence of peptic activity of free and immobilized pepsin at pH 4 0 and various temperatures The immobilized pepsin preparation had high stability at pH 4 0 and 6°C Only 7% loss of activity was observed after 50 days storage Even at pH 4 0 and 37°C, a proper condition for digestion of IgG, the immobilized pepsin was comparatively stable Although rapid inactivation of the immobilized pepsin was observed at 60°C, the half-life of the preparation was still about twice as long as that of free pepsin under the same conditions The immobilization of pepsin on the matrix was, therefore, considered to provide the pepsin molecule with stability against heat-denaturation, autolysis and leakage from the matrix

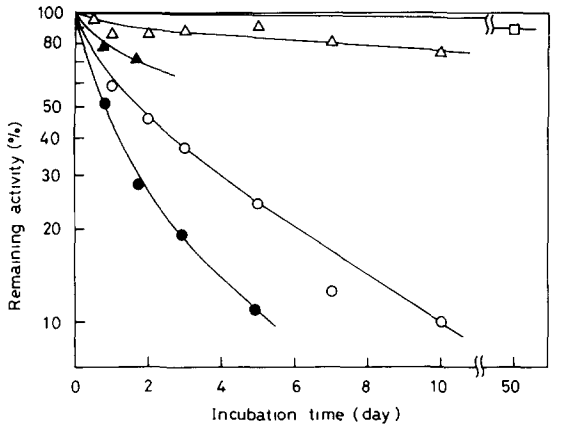


Fig 5 Stability of peptic activities of free and immobilized pepsin at various temperature An immobilized pepsin fixed at the ratio of 3 39 mg/ml gel on the matrix was incubated at 6°C (□), 37°C (△) and 60°C (○) at pH 4 0 Free pepsin was incubated at 37°C (▲) and 60°C (●) at pH 4 0 Peptic activities were assayed using IgG as substrate at pH 4 0

As previously described, glutaraldehyde used in this experiment consisted of mainly oligomer and polymer The oligomeric and polymeric glutaraldehyde species are known to react with amino groups of AH-Sepharose and a lysine residue or the N-terminal of pepsin molecules to form non-conjugated Schiff bases (I) or conjugated Schiff bases (II) as follows

$$OHC\text{-}(CH_2)_3CH{=}\left[C(\text{-CHO})\text{-}(CH_2)_2CH{=}O\right]_n + R\text{-}NH_2$$

Oligomeric and Polymeric glutaraldehyde
R = AH-Sepharose or pepsin

$$R\text{-}N{=}HC\text{-}(CH_2)_3CH{=}\left[C(\text{-CHO})\text{-}(CH_2)_2CH{=}O\right]_n \quad (I)$$

$$OHC\text{-}(CH_2)_3CH{=}\left[C(\text{-CH=N-R})\text{-}(CH_2)_2CH{=}O\right]_n \quad (II)$$

The non-conjugated Schiff base linkage, which is susceptible to acid hydrolysis, may have been hydrolyzed by washing the matrix with 0 01 M HCl Therefore, pepsin molecules might be finally fixed by the conjugated Schiff base linkage, which could be stable in the acid The stable linkages thus formed were considered to prevent pepsin molecule from leaking from the matrix

*Limited cleavage of IgG by immobilized pepsin* Fig 6 shows the time-dependent high-performance

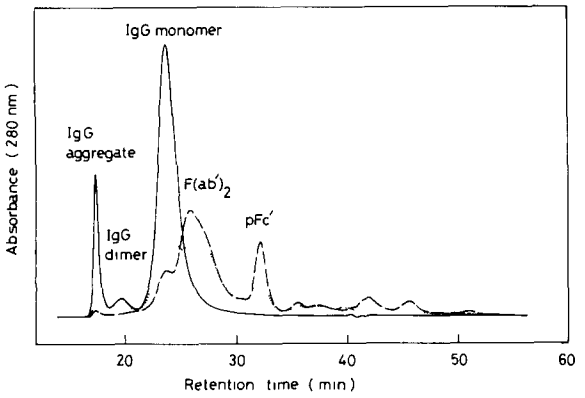


Fig 6 High-performance liquid chromatograms of native and peptic digested IgG Digestions of IgG (20 mg/ml) with free pepsin (1 wt % against IgG) were carried out in 0 1 M acetate buffer (pH 4 0)/0 15 M NaCl at 37°C ———, native IgG, ------, digests after 15 min, – – –, digests after 60 min

liquid chromatograms of IgG digested with pepsin The commercial IgG containing aggregates and dimer as well as monomer, was digested to produce the $F(ab')_2$ fragment of molecular weight 1 02 $10^5$, pFc' fragment of 3 8 $10^4$, and low molecular weight oligopeptides (Tomono, Suzuki, and Tokunaga, unpublished data) The reaction products of the peptic digestion were identical in both free and immobilized pepsin systems

Further investigation was carried out on the limited cleavage of IgG during passage through an immobilized pepsin column, since the column method should provide some advantages in the industrial application of immobilized pepsin as compared with the batch method Fig 7 shows the HPLC diagrams of peptic digests eluted from an immobilized pepsin column (a) and the amounts of four main peptic fragments, $F(ab')_2$, pFc', and two oligopeptides (b) It was of interest to note that the $F(ab')_2$ fragment of higher molecular weight was eluted through the immobilized pepsin column faster than the other low molecular weight fragments, i e , pFc', peptide A and B The elution time of the $F(ab')_2$ fragment was about 89% that of peptide B This result indicated that the peptic fragments seemed to be subjected to gel-filtration by the immobilized pepsin gel Therefore, the pure $F(ab')_2$ fragment was observed particularly in the initial fractions eluted from the immobilized pepsin column This

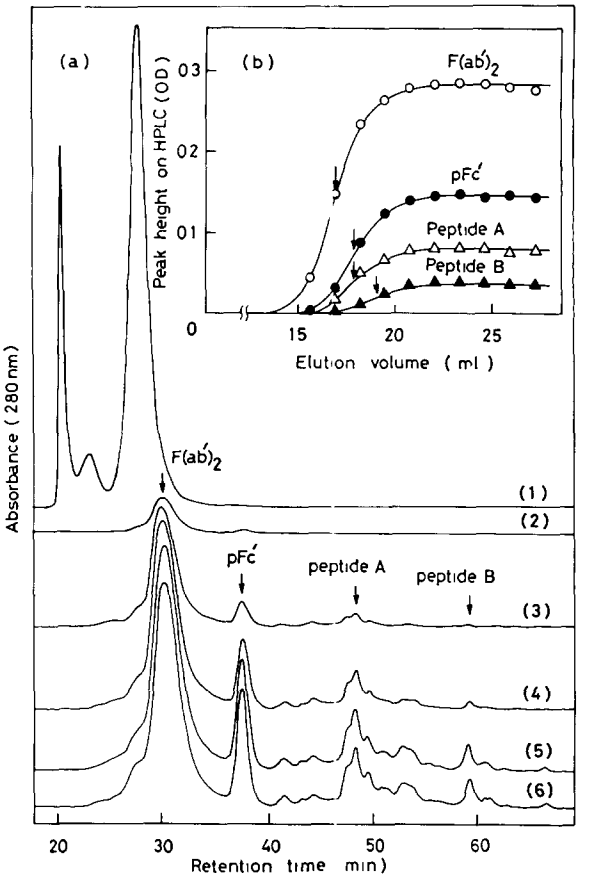


Fig 7 High-performance liquid chromatograms of peptic digests eluted from an immobilized pepsin column (a) and the elution profiles of major peptic fragments, $F(ab')_2$, pFc', and two oligopeptides, with time (b) The chromatograms shown in a were that of IgG before digestion (1) and that of the fractions at 15 6 (2), 16 9 (3), 18 2 (4), 20 8 (5), and 26 0 (6) ml of elution from an immobilized pepsin column (pepsin fixed at the ratio of 2 34 mg/ml gel) The arrows in b indicated the elution volume at which the peak height of each peptic fragment corresponded to 50% of equilibrium peak height

effect should be applicable to produce intravenous preparations of $F(ab')_2$ fragment industrially with the advantage that the pure $F(ab')_2$ fragment could be obtained by a one-step procedure without further purification after digestion

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