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Single-step Purification of Pepsin-derived Monoclonal Antibody Fragments from Crude Murine Ascitic Fluids by Ceramic Hydroxyapatite High-performance Liquid Chromatography

Ayaka Moro[1], Tomohiko Yoshitake[2], Tetsuro Ogawa[2,*] and Tohru Ichimura[1,†]

[1]Department of Applied Chemistry, National Defense Academy, Yokosuka, Kanagawa 239-8686; and [2]R&D Department of New Ceramic Division, Life Care Business Division, PENTAX Corporation, Itabashi-ku, Tokyo 174-8639, Japan

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Ceramic hydroxyapatite (CHT) high-performance liquid chromatography (HPLC) is used to purify a variety of classes of monoclonal antibodies (mAbs) from crude murine ascites fluids. We report here that this method is also applicable for simple and efficient purification of many mAb fragments that are generated by pepsin treatment of crude ascites. $F(ab')_2$ fragments were quantitatively generated from IgG_1 mAbs in ascitic fluids by incubation with pepsin for 6 h at pH 3.9–4.1. Under the same conditions, pepsin also cleaved unwanted ascites components, such as albumin and transferrin to very low molecular weight polypeptides. The $F(ab')_2$ fragments, but not the low molecular weight products, selectively bound to and were eluted from the CHT column using a linear gradient of phosphate ion concentration over 15 min. The recovery of the $F(ab')_2$ fragments by CHT-HPLC was >90%. This method also allowed single-step purification of mAb fragments from distinct IgG subclasses (IgG_{2a} and IgG_{2b}) and IgM directly from crude digested ascitic samples. This CHT-HPLC method combined with direct pepsinolysis of murine ascites is a useful strategy for rapid purification and characterization of many types of mAb fragments.

Key words: antibody fragment, ceramic hydroxyapatite, high-performance liquid chromatography, monoclonal antibody, purification.

Abbreviations: CHT, ceramic hydroxyapatite; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody.

Monoclonal antibodies (mAbs) are increasingly being used as probes for research in basic biology and biopharmaceuticals. They are also used for immunodiagnosis and immunotherapy for several human diseases including cancers. Preparations of mAb fragments are sometimes needed, for example, to avoid potential non-specific binding of intact mAb molecules to constant region (Fc) receptors on lymphocytes. Antibody fragments usually retain the antigen-binding activity of the original mAb and the inhibitory effects against a toxin or microbe, but the fragments do not have the Fc region and thus are not susceptible to Fc-associated effector functions or potential non-specific binding.

Several high-performance liquid chromatography (HPLC) systems have been introduced as the preferred method for purification of antibody fragments that are prepared by enzymatic digestion of whole immunoglobulins or recombinantly in *Escherichia coli* or other host cells (*1–5*). These systems include size-exclusion HPLC, ion-exchange HPLC and hydrophobic interaction HPLC. However, the protocols described therein generally involve numerous steps and require a lot of time. Furthermore, most of the published protocols use salt precipitation prior to HPLC purification, which requires an additional step to perform on a large scale under sterile conditions (e.g. sterile filtration) and may result in mAb and antibody fragment loss.

Recent studies have shown that ceramic hydroxyapatite (CHT)-HPLC is broadly applicable for purification of all classes of mAbs secreted into ascites and cell culture supernatants (*6–12*). For example, Yamakawa and Chiba (*6*) demonstrated that 16 mAbs, including all four subclasses of IgG and IgM, could be purified from crude murine ascitic fluids by CHT-HPLC in a single step. Bukovsky and Kennett (*7*) also reported that CHT-HPLC adequately purified c-myc mAb (termed Myc-x-5-1) directly from cell culture supernatants. Importantly, several papers have noticed that each mAb has a different retention time on CHT-HPLC, even within the same immunoglobulin class (*6*, *13*), suggesting that CHT-HPLC may separate mAbs through interaction with their variable/antigen-binding fragment (Fab) regions rather than through the constant Fc region probably by zwitter ion-type retention mechanism (*14*). In the present study, we describe the use of CHT-HPLC to purify Fab fragments directly from pepsin-treated crude murine ascitic fluids.

*Present address: Laboratory Skin Care Inc., Taboe City, CA 96145-7469, USA.
†To whom correspondence should be addressed. Tel: +81-468-41-3810 (ext. 3563), Fax: +81-468-41-5901,
E-mail: ichimura@nda.ac.jp

MATERIALS AND METHODS

Materials—Murine ascites containing MOPC-21 (IgG_1, κ), UPC-10 (IgG_{2a}, κ), MOPC-141 (IgG_{2b}, κ) and MOPC-104E (IgM, λ) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The ascites containing 9E10 (IgG_1) and SUK4 (IgG_1) were produced in BALB/c mice using conventional procedures (*15*). Pepsin (EC 3.4.23.1) was of recrystallized grade and was obtained from Nacalai tesque (Kyoto, Japan). Mouse transferrin and albumin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Unless noted, other materials used in this study were of analytical grade from Wako Pure Chemical Industries.

Pepsinolysis—Each ascites sample was dialysed against 0.1 M sodium acetate buffer with the indicated pHs (see figure legends), and the protein concentration was adjusted to 27–106 mg/ml (mAb concentration of 0.4 mg/ml). Pepsin was then added to the solution at a final enzyme : mAb ratio of 1 : 33 (w/w), and the mixture was incubated for 6 h at 37°C. Before analysis, the reaction was stopped by adding an equal volume of 2 M Tris–HCl, pH 8.8, to give a pH around 7.

CHT-HPLC—The HPLC apparatus consisted of two pumps (Shimadzu, Kyoto, Japan, LC-6A), one programmer (Shimadzu, SCL-6A), one UV absorbance detector (Shimadzu, SPD 10AV), one injection valve (Rheodyne, Theale, Berkshire, UK, type 7125) with 1-ml sample loop (Rheodyne, type 7027) and one recorder (Windows PC with a signal converter). CHT-HPLC was performed on a CHT column (4.6 × 35 mm I.D., particle size 10 μm; Pentax, Tokyo, Japan). Proteins or pepsin-digested ascites samples (10–50 μg of mAb) were loaded on the CHT column equilibrated with Buffer A (5 mM sodium phosphate, pH 6.8) and eluted by a 15-min linear gradient of Buffer B (400 mM sodium phosphate, pH 6.8) at 1 ml/min.

Analytical Studies—SDS–PAGE was carried out essentially according to the procedure of Laemmli (*16*) using 10% polyacrylamide gel under non-reducing conditions. Proteins were stained by Coomassie brilliant blue R-250. When necessary, proteins were precipitated using the method of Wessel and Flugge (*17*) prior to SDS–PAGE. Protein concentration was determined according to Bradford (*18*) using cattle serum IgG as a standard. The percentage of recovery of $F(ab')_2$ fragments released from mAb after pepsinolysis was calculated by densitometric quantification of the Coomassie-stained bands of mAb and $F(ab')_2$ fragments on the SDS–PAGE gel and by assuming that M_r is 170-kDa for IgG and 110-kDa for the $F(ab')_2$ fragments. The recovery of $F(ab')_2$ fragments following CHT-HPLC was determined according to Yamakawa and Chiba (*6*) by comparing the peak areas before and after re-chromatography.

RESULTS

Determination of Standard Conditions for Pepsinolysis of Murine Ascites—Although several procedures have been reported for the direct pepsinolysis of ascites fluids, they vary widely in buffer conditions and generally require a long incubation time (24–72 h). To improve these conditions, the buffer pH and incubation time were optimized along with use of a relatively high concentration of pepsin [enzyme : substrate (E : S) = 1 : 33 (w/w)] and two kinds of murine ascites, both of which contained the most common immunoglobulin class IgG_1 (termed MOPC-21 and 9E10), as models (see also MATERIALS AND METHODS section). Figure 1 shows the effect of buffer pH on the production of $F(ab')_2$ fragments following a 6-h incubation with pepsin, as analysed by SDS–PAGE

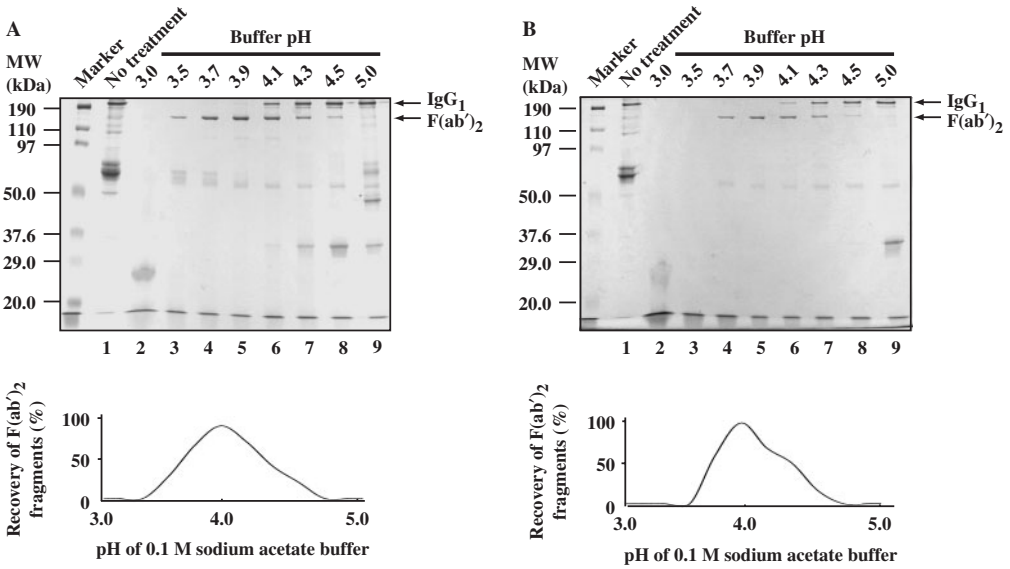

Fig. 1. **Effect of pH on direct pepsinolysis of murine ascites.** Murine ascites containing monoclonal antibody MOPC-21 (A) and 9E10 (B). Upper panels, each ascites sample was digested by pepsin in 0.1 M sodium acetate buffer at pH 3.0–5.0 for 6 h at 37°C (lanes 2–9). The control experiment was done as above at pH 3.9 without pepsin (lane 1). After digestion, each digest (~10 μg of protein) was analysed by 10% SDS–PAGE under non-reducing conditions. Lower panels, the extent of $F(ab')_2$ fragmentation produced by pepsin digestion was also quantified by densitometry and is presented as a percentage of the theoretical yield by assuming that M_r is 170-kDa for IgG1 and 110-kDa for the $F(ab')_2$ fragments. Similar results were obtained from at least three independent experiments.

under non-reducing conditions. With both of the IgG_1 samples, the $F(ab')_2$ fragments were readily produced/recovered in buffer of pH ~4.5 (Fig. 1A and B; lane 8), and the recovery increased as the buffer pH was decreased, although fewer (or no) $F(ab')_2$ bands were detected at pH 3.0–3.5 (lanes 2–3). The production of $F(ab')_2$ fragments was also confirmed by western blotting with antibodies specific to $F(ab')_2$ (data not shown). Densitometric quantitation of the corresponding $F(ab')_2$ bands revealed that the highest yield of $F(ab')_2$ production (>90%) was achieved at pH 3.9–4.1 (Fig. 1, lower panels). Similar experiments carried out for different incubation times (3–48 h, at pH 3.9) showed that incubation < 6 h (e.g. 3 h or 4 h) significantly reduced the $F(ab')_2$ production (to 70–80% yield), whereas longer incubation (e.g. 12 h, 24 h or 48 h) did not improve the yield (data not shown). Therefore, we established our standard pepsinolysis conditions as incubation for 6 h in a buffer at pH 3.9–4.1 with an E : S ratio of 1 : 33.

Murine ascitic fluids contain large amounts of various non-immunoglobulin proteins such as albumin and transferrin [(*6*); see also, Fig. 1A and B; lane 1]. However, under the standard conditions described above, pepsin also cleaved these unwanted ascites components into low molecular weight (< 20 kDa) polypeptides, although the $F(ab')_2$ molecules were left intact (Fig. 1A and B; lane 5). To confirm this result and further refine the standard condition, purified preparations of immunoglobulins (MOPC-21 and 9E10), transferrin and albumin were incubated directly with pepsin as above (at pH 3.9). As expected, transferrin and albumin, but not $F(ab')_2$ fragments from MOPC-21 and 9E10, were indeed degraded into small peptides migrating with the buffer front on an SDS–PAGE gel (Fig. 2, lanes 6 and 8).

Purification of mAb Fragments by CHT-HPLC— Previous studies have shown that mAbs do not elute from a hydroxyapatite column below 5–10 mM phosphate buffer, whereas all mAbs elute from the column below 300–400 mM phosphate buffer (*6*, *7*, *14*, *19*, *20*). Therefore, we chose 5 mM sodium phosphate as the initial electrolyte concentration and 400 mM as the final electrolyte concentration in the present study assuming that such buffer conditions might also be suitable for the systematic purification of many types of mAb fragments. The pepsin digests of MOPC-21 and 9E10 ascites were directly loaded onto the CHT column and separated using a 15-min linear gradient of 5–400 mM phosphate at pH 6.8. Figure 3A shows typical elution profiles on CHT-HPLC of MOPC-21 and 9E10 ascites incubated without pepsin (left panels) or with pepsin (right panels). In the undigested samples, two major and sharp peaks were reproducibly observed in addition to the flow-through (left panels). SDS–PAGE analysis indicated that the first major peak (fractions collected at 4.4–5.2 min) contained albumin as a major constituent, whereas the second peak (fractions collected at 5.2–6.6 min for MOPC-21 and 5.9–7.2 min for 9E10) corresponded to mAb (data not shown). These elution profiles were very similar to those reported by Yamakawa and Chiba (*6*). With the digested samples, however, only one major and sharp peak was detected under the same elution condition, consistent with the loss of the albumin peak, in addition to an increased amount of flow-through (right panels). The peak eluates (fractions collected at 4.2–5.6 min for MOPC-21, 5.5–6.9 min for 9E10) were analysed by SDS–PAGE. This analysis revealed that the $F(ab')_2$ fragments produced from either of the two IgG_1 mAbs were purified to homogeneity only after this single HPLC step (Fig. 3B, lanes 3 and 6). Unwanted components originally present in the digested samples such as the low molecular weight digests and a ~60-kDa polypeptide (Fig. 3B, lanes 2 and 5; marked by asterisk) were found in the flow-through fraction and thus separated completely from the $F(ab')_2$ (as analysed by SDS–PAGE, data not shown). To determine the recovery of $F(ab')_2$ fragments from the CHT column, the purified $F(ab')_2$ were chromatographed to compare the values of $A_{280\,nm}$ before and after re-chromatography. The recovery of $F(ab')_2$ fragments using this chromatography was estimated as 90% for MOPC-21 $F(ab')_2$ and 91% for 9E10 $F(ab')_2$ (Table 1). Incidentally, both the $F(ab')_2$ fragments re-chromatographed were eluted as sharp peaks with identical retention times shown in Table 1.

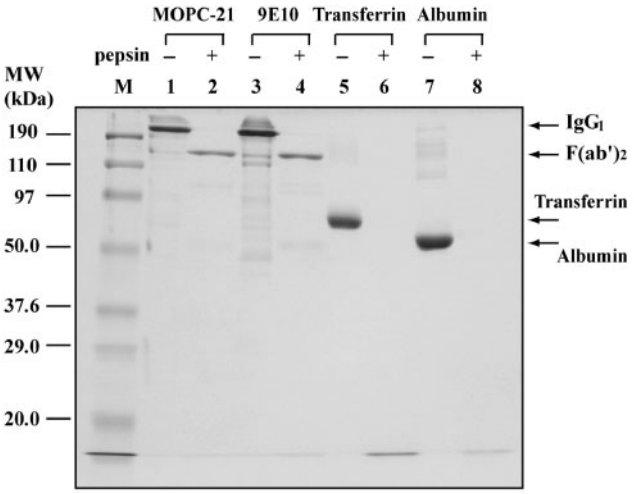

Fig. 2. **SDS–PAGE profiles of protein samples after digestion with pepsin.** MOPC-21 and 9E10 (purified from the ascites by precipitation with ammonium sulphate), and mouse serum transferrin and albumin were incubated with (lanes 2, 4, 6 and 8) or without pepsin (lanes 1, 3, 5 and 7) in 0.1 M sodium acetate buffer (pH 3.9) and then analysed by SDS–PAGE as in Fig. 1. In each lane, ~2 μg of protein was loaded on the gel.

Purification of Other mAb Fragments by CHT-HPLC— To evaluate the availability of the present system, three additional murine ascites samples that contained either mAb IgG_1 (termed SUK4), IgG_{2a} (termed UPC-10) or IgG_{2b} (termed MOPC-141) were digested with pepsin as above and then analysed by SDS–PAGE and CHT-HPLC under the same conditions. Figure 4 (lanes 2, 5 and 8) shows the proteolytic products resolved by SDS–PAGE. In general, a good efficiency of fragmentation was observed commonly for all of these ascites samples, although the IgG_2 mAbs (UPC-10 and MOPC-141) were relatively sensitive to pepsin hydrolysis (Table 1). When these IgG_2 molecules were digested, however, it was evident that some or all the fragment products were converted into Fab′-like fragments with molecular weight

of 50–60 kDa (lanes 5 and 8), not into $F(ab')_2$. This observation is consistent with previous reports (*21*, *22*) and is probably due to the fact that pepsin preferentially cleaves IgG_2 molecules at the hinge region N-terminal to the inter-heavy chain disulphide bonds. We found, however, that all the antibody fragments generated, including the Fab′-like fragments of UPC-10 and MOPC-141, could also be purified from other contaminants by CHT-HPLC in a single step (chromatograms not shown). The SDS–PAGE analysis of purified antibody fractions is presented in Fig. 4 (lanes, 3, 6 and 9). The $F(ab')_2$ and Fab′-like fragments of UPC-10 eluted from the CHT column with very close retention times. Thus, separation of these fragments using the present elution system was difficult (Fig. 4, lane 6, see also DISCUSSION section). The recoveries of the pepsin-generated mAb fragments following CHT-HPLC were all >90% (as assessed by re-chromatography), similar to those observed for the MOPC-21 and 9E10 $F(ab')_2$ samples (Table 1).

Purification of IgM $F(ab')_{2\mu}$ fragments by CHT-HPLC—IgM contains five four-chain base units (two light chains and two heavy chains) that are connected via disulphide bonds. The molecular weight of this immunoglobulin class is about 1,000 kDa. IgM is included a lot next to IgG in blood; however, because of its large size and molecular bulkiness, the number of potential applications of IgM is restricted, particularly in immunohistochemical studies. We digested the murine ascites sample containing mAb IgM (termed MOPC-104E) with pepsin as above, and separated the proteins using CHT-HPLC. Figure 5A shows the elution profiles on CHT-HPLC of the MOPC-104E ascites incubated in the absence (left panel) or presence (middle panel) of pepsin. In the undigested sample, two major and sharp peaks (the first and second peaks correspond to Alb and IgM, respectively, as analysed by SDS–PAGE) were reproducibly detected by CHT-HPLC (left panel), whereas in the digested sample, relatively broad and

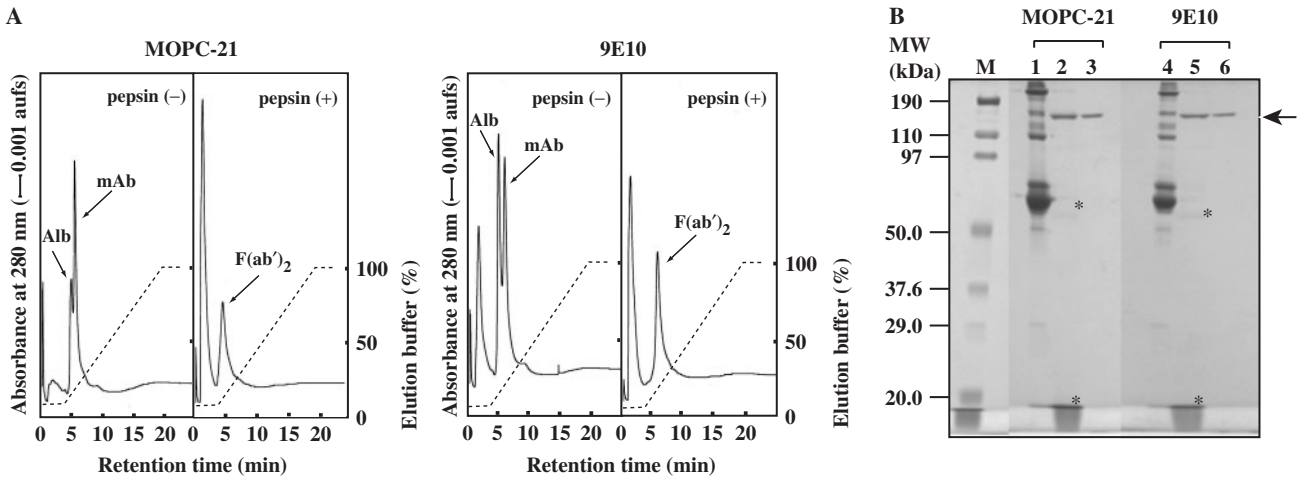

Fig. 3. **Purification by CHT-HPLC of $F(ab')_2$ fragments of MOPC-21 and 9E10 produced by pepsin digestion.** (A) Murine ascites containing monoclonal antibody MOPC-21 or 9E10 (~50 μg protein each) incubated without pepsin (left panels) or with pepsin (right panels) at pH 3.9 were separated by CHT-HPLC with a 15-min linear gradient of phosphate buffer (pH 6.8, 5–400 mM) at a flow-rate of 1.0 ml/min. aufs, absorbance units full scale. (B) The $F(ab')_2$ eluates (right panels, fractions collected at 4.2–5.6 min for mAbs (MOPC-21 and 5.5–6.9 min for 9E10) were analysed by SDS–PAGE (100 μl each, lanes 3 and 6) as in Fig. 1. The MOPC-21 and 9E10 ascites before (lanes 1 and 4) and after (lanes 2 and 5) pepsinolysis were also run in parallel to compare the protein content (~10 μg of protein each). The miscellaneous components originally present in the digested samples (i.e. the low molecular weight digests and ~60-kDa polypeptide) are indicated by asterisks. The arrow indicates the position of $F(ab')_2$ fragments.

Table 1. **mAb antibody fragments characterized in this study.**

mAbs		Pepsinolysis		CHT-HPLC		
Class or subclass	Name of mAb	Primary product(s) after pepsinolysis	Recovery of mAb fragments (%)[a]	Retention time (min)	Phosphate ion concentration (mM)	Recovery of mAb fragments (%)[b]
IgG_1	MOPC-21	$F(ab')_2$	93	4.6	8.8	90
IgG_1	9E10	$F(ab')_2$	96	5.9	16	91
IgG_1	SUK4	$F(ab')_2$	96	6.4	20	92
IgG_{2a}	UPC-10	$F(ab')_2$, Fab′	47	6.9	23	91
IgG_{2b}	MOPC-141	Fab′	50	8.9	36	90
IgM	MOPC-104E	$F(ab')_{2\mu}$	87	10.5[c]	15	91

[a]Recovery rate was determined by densitometric quantitation of the Comassie blue-stained bands of mAb and its antibody fragments after SDS–PAGE. [b]Recovery rate was determined by calculating the peak areas of the antibody fragments before and after re-chromatography. [c]Phosphate gradient was started 5 min after sample injection.

irregular peaks resolved into four close peaks were observed under the same elution conditions (middle panel). The eluates of the digested sample were collected every 30 s and then analysed by SDS–PAGE. The $F(ab')_{2\mu}$ fragments with a M_r of ~140 kDa eluted over a narrow range of the gradient, corresponding exactly to the observed second and third sharp peaks (Fig. 5A, middle panel; data not shown). The $F(ab')_{2\mu}$ peaks were pooled and re-examined by SDS–PAGE, which clearly indicated that the purified pool contained only $F(ab')_{2\mu}$ (Fig. 5B, lane 3). The recovery of $F(ab')_{2\mu}$ during CHT-HPLC was 91% as assessed by re-chromatography (Table 1).

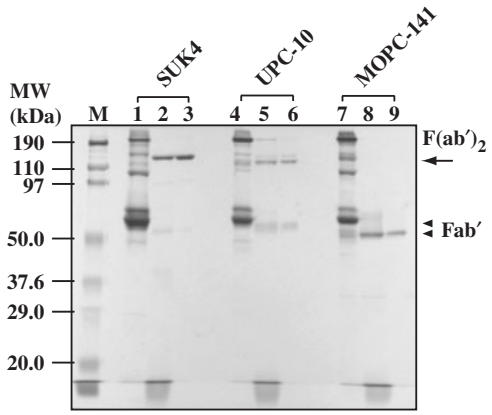

Fig. 4. **Purification of antibody fragments of SUK4 (IgG_1), UPC-10 (IgG_{2a}) and MOPC-141 (IgG_{2b}) by CHT-HPLC.** Murine ascites containing SUK4, UPC-10 and MOPC-141 were incubated without pepsin (lanes 1, 4 and 7) or with pepsin (lanes 2, 5 and 8) at pH 3.9, and the antibody fragments were separated by CHT-HPLC (lanes 3, 6 and 9) and analysed by SDS–PAGE as in Fig. 1. The arrow and arrowheads indicate the positions of $F(ab')_2$ and F(ab′)-like fragments, respectively.

We note, however, that the purified $F(ab')_{2\mu}$ was still separated into two close peaks as analysed by re-chromatography (Fig. 5A, right panel), although no residual protein was detected in an SDS–PAGE gel of the corresponding fraction (Fig. 5B, lane 3). Such elution of $F(ab')_{2\mu}$ from the CHT column might reflect the reported heterogeneity of the $F(ab')_{2\mu}$ species (*23*, *24*) that results from differences in carbohydrate content or the difference in the relative positions in immunoglobulin heavy chains that may affect susceptibility to cleavage by pepsin.

DISCUSSION

This article describes the use of CHT-HPLC for simple and efficient purification of mAb fragments that are generated by pepsin treatment of crude murine ascitic fluids. The most important feature of this method is that the digested samples can be separated directly without pre-treatment, e.g. salt precipitation or dialysis. This is clearly advantageous compared to several conventional methods using size-exclusion HPLC, ion-exchange HPLC and/or hydrophobic interaction HPLC (*1–5*), because less time is required (total purification time, 7–8 h) and sample-handling is reduced (only pepsinolysis and CHT-HPLC). In addition, the excellent capacity of CHT-HPLC, good chromatographic resolution and high protein recovery enabled single-step purification of all antibody fragments tested, including those from IgG_1, IgG_{2a}, IgG_{2b} and IgM directly from the crude digested ascites samples (Figs 3–5). We also examined the CHT gel matrix for deterioration after consecutive runs with the 9E10 digests. Even after 30 cycles of chromatography, no significant change was observed in retention time, flow pressure, the shape of the peak or recovery of the corresponding $F(ab')_2$ fragments (data not shown), indicating that the CHT matrix is also stable for

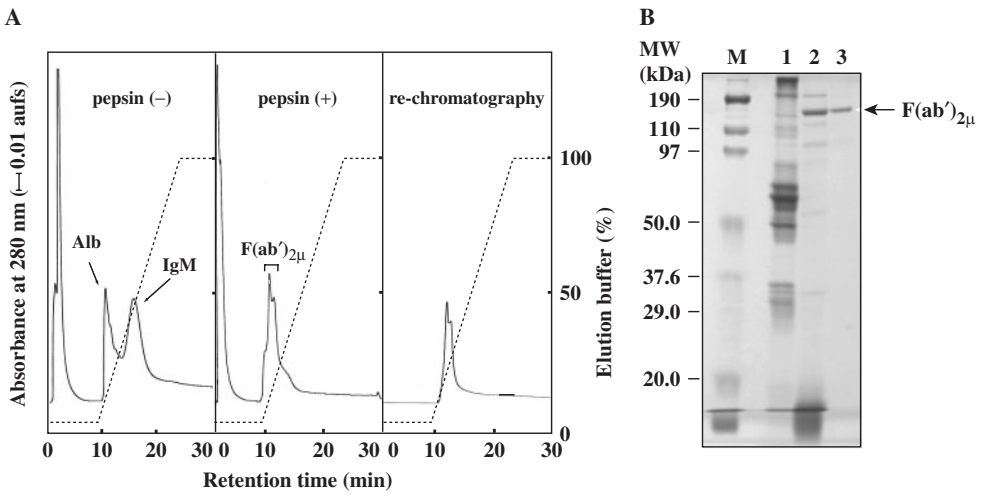

Fig. 5. **Purification of $F(ab')_{2\mu}$ fragments of mAb IgM by CHT-HPLC.** (A) Murine ascites samples containing MOPC-104E (IgM) incubated without pepsin (left panel) or with pepsin (right panel) at pH 4.1 were separated by CHT-HPLC with a 15-min linear gradient of phosphate buffer (pH 6.8, 5–400 mM) as in Fig. 3A (~500 μg of protein each). The $F(ab')_{2\mu}$ eluate [marked in the middle panel of (A), fractions 10–12 min] was collected and analysed by re-chromatography (right panel). aufs, absorbance units full scale. (B) The MOPC-104E ascites incubated without pepsin (lane 1) or with pepsin (lane 2) (~10 μg of protein each) and the $F(ab')_{2\mu}$ fragments separated by CHT-HPLC (fractions 10–12 min; lane 3, 20 μl) were analysed by SDS–PAGE as in Fig. 1.

repeated runs under the experimental conditions described here. These findings suggest that this CHT-HPLC method combined with direct pepsinolysis of murine ascites is a useful strategy for rapid purification and characterization of many mAb fragments.

Each mAb and its antibody fragments have different retention times on CHT-HPLC even within the same class of immunoglobulin [Table 1; (*8*, *14*)]. This retention behaviour is clearly different from that observed with other HPLC systems (*14*) and suggests strongly that CHT-HPLC separates mAbs through interaction with their variable/antigen-binding fragment (Fab) regions rather than through the constant Fc region. Although with recombinant production of antibody fragments there is no special need to discriminate between Fab and Fc, the separation property of CHT-HPLC may be of practical important. Indeed, production of Fab fragments from whole immunoglobulins is increasingly popular in the fields of basic biology and pharmaceuticals and is being performed on both analytical and preparative levels using several conventional HPLC systems. Therefore, we suggest that CHT-HPLC is also applicable as an alternative or compensatory method when other HPLC systems are not suitable for efficient purification of desired antibody fragments.

Although we note that the purified $F(ab')_{2\mu}$ fragments derived from IgM mAb (MOPC-104E) were still separated into two close peaks by re-chromatography on a CHT column (Fig. 5A, right panel), this was not unique to fragments from this antibody type. Indeed, Mochizuki *et al*. (*23*) reported that $F(ab')_{2\mu}$ fragments prepared from mAb HB4C5 were resolved into two close fractions by chromatography on hydroxyapatite. There is heterogeneity in sugar content between many mouse IgM or IgG mAbs generated by hybridoma technology that appears to be more extensive in IgM than IgG because of its much larger molecular size and pentameric structure (*23–26*). It was also reported that IgM mAbs have multiple sites within the heavy chains that are sensitive to pepsin digestion (*27*). Thus, the observed split elution of $F(ab')_{2\mu}$ fragments from the CHT column might reflect such structural heterogeneity of $F(ab')_{2\mu}$ species, e.g. the difference in carbohydrate content and/or the difference in the positions in the heavy chains cleaved by pepsin. Future studies will be required to explore this hypothesis.

Yamakawa and Chiba (*6*) showed that every ascitic fluid sample contains a small amount of non-specific polyclonal antibodies derived from the host cells and that such polyclonal antibodies elute from a CHT column over a broad range of the gradient. This implies that the purified fractions of our mAb fragments also should contain a low level of host cell-derived antibody fragments. Thus, serum-free culture medium instead of ascites fluids should be used if such residual antibody fragments need to be avoided. Under the chromatographic conditions we used, fragments that had quite similar retentions times, such as the $F(ab')_2$ and Fab′-like fragments of UPC-10, were only minimally separated. Thus, modifications such as changing the pH of the eluent or using a more shallow phosphate gradient may be necessary to completely separate antibody fragments having similar retention times.

In summary, our results show that many types of mAb fragments are successfully purified from crude digested ascites samples by CHT-HPLC in a single step. Because all the procedures described herein can be carried out within half a day, this method is particularly useful when time and cost are important factors for preparing the desired mAb fragments.

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CONFLICT OF INTEREST

None declared.

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