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Enzyme-linked immunosorbent assay-based selection and optimization of elution buffer for TAG72-affinity chromatography

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Abstract

An enzyme-linked immunosorbent assay (ELISA)-elution assay was developed to screen a large variety of elution buffers for selection of a suitable one for purification of the fusion protein FV/TNF-α by affinity chromatography. Various commonly used buffer systems utilizing widely differing conditions such as extreme pH, denaturants, chaotropic ions and polarity reducing reagents were investigated. Ammonia solution (1 *M*, pH 11.5) proved to exert the most suitable influence on dissociation of the FV/TNF-α/TAG72 complex while having a minimal protein denaturing effect on FV/TNF-α. The total yield of purified FV/TNF-α using the TAG72-affinity column with this elution system was 300-fold higher than that using the common elution buffer, 0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7. Our study indicates that the ELISA-elution assay will be most useful in the selection of suitable elution buffers for affinity chromatography.

Keywords: ELISA; Elution buffers; TAG72; Proteins

1. Introduction

Affinity chromatography for isolation of biomolecules and/or their ligands is highly efficient in terms of both specificity and capacity. It can achieve a 2000- to 20 000-fold purification factor in a single step. Furthermore, coupling of a monoclonal antibody onto an affinity column increases biological activity. The limitations of this powerful purification procedure are complete dissociation of analyte from the affinity matrix at the cost of losses in biological activity or decreases in analyte yield while conserving its full biological activity. Therefore, the search for an ideal dissociating reagent becomes a central task in affinity chromatography [1]. However, most available methods having been used to assess the dissociation potency of elution buffer candidates are associated with severe analyte loss, were laborious and suffered from problems in adjusting elution buffer concentrations [2,3]. These difficulties frequently hampered the application of this otherwise

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simple and efficient purification procedure in practice.

The forces contributing to antigen–antibody interactions involve electrostatic, hydrophobic, van der Waals and hydrogen bonding [4]. Therefore, an effective eluent should influence either all or a majority of these four types of interactions. Many chemicals are effective in disrupting these forces and can be categorized according to their physicochemical properties: (i) extreme pH, which weakens all the forces except hydrophobic interactions; (ii) chaotropic salts, which influence the structure of the surrounding water layer and thereby indirectly affect hydrophobic interactions; (iii) ionic strength, which disrupts the charge–charge interactions; (iv) denaturants, which melt off the protein structures and (v) organic solvents, which are polarity reducing agents [1,5]. Since the contribution of each interaction force differed in each individual ligand–counter ligand complex system, it is difficult to apply an universal applicable eluent. In fact, some eluent system could cause dramatic changes in elution profiles when applied to monoclonal [6] as well as polyclonal antibodies [2,7]. Therefore, each application requires its own ''inherent'' elution system.

We have recently expressed a secreted fusion protein FV/TNF-α containing the FV fragment of the B72.3 antibody recognizing the tumor-associated TAG72 antigen and tumor necrosis factor alpha (TNF-α) in *E. coli* culture supernatant [8]. The fusion protein FV/TNF-α (M_r 43 000) was further purified from the *E. coli* supernatant by TAG72-affinity chromatography for use in targeting TNF-α to tumors expressing the TAG72 antigen for tumor immunotherapy. However, most of FV/TNF-α eluting from the TAG72-affinity column using the common eluent (0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7) [5] was denatured by protein aggregation. In this study, we screened a large variety of different eluents for their ability to dissociate complexes formed between the FV fragment of the FV/TNF-α and the TAG72 antigen. A modified enzyme-linked immunosorbent assay (ELISA), termed ''ELISA-elution assay'' was applied for screening the optimized elution conditions, which was further utilized for purification of the fusion protein FV/TNF-α using TAG72-affinity chromatography.

2. Materials and methods

2.1. Antibodies, antigen, microtiter plates and chemicals

The purified rabbit polyclonal anti-TNF-α antibody and the peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) antibody were obtained from Gibco (Burlington, Canada) and Bio/Can Scientific (Mississauga, Canada), respectively. Bovine submaxillary mucin (BSM) type I-S which contains the TAG72 epitopes was used as the source of TAG72 antigen and was purchased from Sigma (Oakville, Canada). Ninety six-well polystyrene ELISA plates were purchased from Fisher Scientific (Toronto, Canada). CNBr-activated Sepharose 4B was purchased from Amersham-Pharmacia Biotech (Baie d'Urfe, Canada). The TAG72-affinity column was prepared by conjugating BSM to CNBr-activated Sepharose 4B according to Ref. [9]. All chemicals used for preparation of elution buffers were purchased from Sigma.

2.2. Preparation of fusion protein FV/TNF-α

The single-chain fusion protein FV/TNF-α containing the FV fragment of the B72.3 antibody and the TNF-α moiety was expressed in *E. coli* and secreted into the *E. coli* culture supernatant as previously described [8]. After centrifugation, the clear culture supernatant containing FV/TNF-α was used as the raw source for the screening of elution buffers and for purification of FV/TNF-α using TAG72-affinity chromatography.

2.3. Preparation of elution buffers

Elution buffers were selected based on the five types of commonly used elution conditions: extreme pH, chaotropic salts, ionic strength, denaturants and organic solvents [1] and prepared according to the methods described by Tsang and Wilkins [2]. The composition of buffers is given in Table 1. The pH of these buffers was not adjusted unless specified.

Table 1
Composition of elution buffers

Classification	Code of buffer	Composition
Denaturants	A1	8.0 *M* urea
	A2	6.0 *M* guanidine HCl, pH 7.2
Extreme pH	B1	0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7
	B2	0.1 *M* citric acid, pH 2.5
	B3	1.0 *M* sodium carbonate, 0.5 *M* NaCl, pH 11.5
	B4	1.0 *M* ammonia solution, pH 11.5
Ionic strength	C1	1.3 *M* magnesium chloride
	C2	2.5 *M* magnesium chloride
	C3	5.0 *M* magnesium chloride
Chaotropic salts	D1	0.3 *M* potassium iodide
	D2	0.6 *M* potassium iodide
	D3	1.3 *M* potassium iodide
	D4	2.5 *M* potassium iodide
	D5	5.0 *M* potassium iodide
	E1	0.2 *M* potassium thiocyanate
	E2	0.4 *M* potassium thiocyanate
	E3	0.9 *M* potassium thiocyanate
	E4	1.8 *M* potassium thiocyanate
	E5	3.5 *M* potassium thiocyanate
Organic solvent	F1	30% ethylene glycol
	F2	70% ethylene glycol
	F3	100% ethylene glycol
Mixture	G1	3.0 *M* magnesium chloride, 25% ethylene glycol, 75 m*M* Hepes, pH 7.2

2.4. ELISA-elution assay

An ELISA-elution assay was performed by incorporation of an elution step and modification of a sandwich ELISA procedure [10] to evaluate the effectiveness of eluents in the dissociation of FV/TNF-α from BSM coated on microtiter plates. Briefly, 200 ng of BSM was coated to each well of the microtiter plate and incubated overnight at 4°C. Uncoupled active sites on the plate's surface were blocked with bovine serum albumin (BSA) (3%) for 1 h at 37°C in order to reduce non-specific binding of FV/TNF-α. For determination of FV/TNF-α still remaining bound by BSM after incubation with elution buffer, the following procedure was used. Fifty microliters of the *E. coli* supernatant containing the FV/TNF-α and its two-fold serial dilutions were added to BSM-coated wells in triplicate. After incubation for 1 h at 37°C, the supernatants were removed and 100 μl of each elution buffer was added for incubation at room temperature for 20 min. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.5% BSA. The bound FV/TNF-α which remained on the plates was detected using rabbit anti-TNF-α antibody (10 μg/ml) followed by donkey anti-rabbit IgG antibody conjugated with peroxidase (1:1000). After another three washes with PBS, 50 μl of substrate buffer containing 0.1 mol/l sodium citrate, pH 6.0, 0.1 mg/ml tetramethylbenzidine and 0.005% H_2O_2 was added to each well of the plate for generation of color reaction. The optical densities were determined at 415 nm in a Bio-Rad Model 3550 microplate reader and compared to the standard FV/TNF-α binding curve which was generated to quantitate the bound FV/TNF-α. For determination of the total

amount of BSM-bound FV/TNF-α, the *E. coli* supernatant containing FV/TNF-α and its two-fold dilutions were added to BSM-coated wells. After incubation for 1 h at 37°C, the plate was washed three times with PBS. Rabbit anti-TNF-α antibody was added to wells, followed by donkey anti-rabbit IgG antibody conjugated with peroxidase. The color reaction was then generated by adding the substrate as described above for generation of the standard FV/TNF-α binding curve.

2.5. Possible coated-BSM removal by elution buffers

To examine whether the elution buffers strip off the BSM bound on the plate, we performed another modified ELISA. Briefly, each well of the microtiter plate was coated with 200 ng of BSM. Various elution buffers were then added to the plates and incubated for 30 min at room temperature. The plates were washed three times with PBS and re-blocked with 3% BSA. The BSM which remained on the plate was incubated with the *E. coli* supernatant containing FV/TNF-α followed by rabbit anti-TNF-α antibody. After three washes with PBS, donkey anti-rabbit IgG antibody conjugated with peroxidase was added to the plate. After another three washes with PBS, substrate was added to the plate for generation of color reaction as described above. The optical densities were determined as described above and compared to the standard TAG72-binding curve which was generated to quantitate the bound BSM on the plate. Briefly, 200 ng of BSM and its two-fold serial dilutions in triplicate were coated to wells of the microtiter plate. After blocking with 3% BSA, *E. coli* supernatant containing FV/TNF-α was added to each well and incubated for 1 h at 37°C. After three washes with PBS, rabbit anti-TNF-α antibody was added to the plate, followed by donkey anti-rabbit IgG antibody conjugated with peroxidase. The color reaction was generated by adding substrate as described above for generation of the standard TAG72-binding curve.

2.6. Denaturation effect of elution buffers

The denaturing effect of elution buffers on the fusion protein FV/TNF-α was also determined by ELISA. The *E. coli* supernatant containing FV/TNF-α was treated with various elution buffers for 30 min at room temperature, and the protein aggregates derived from the protein denaturation were removed by centrifugation at 10 000 *g* after treatment. The elution buffers were removed from the samples by extensive dialysis against PBS and the samples were lyophilized. The samples and their two-fold serial dilutions were added to BSM-coated wells in triplicate. The bound FV/TNF-α was quantitated by adding rabbit anti-TNF-α antibody and donkey anti-rabbit IgG antibody conjugated with peroxidase as described above.

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting analysis

The *E. coli* supernatant containing FV/TNF-α was applied to the TAG72-affinity column for purification of FV/TNF-α. The protein eluted from the column using 1 *M* ammonia solution (NH_4OH), pH 11.5 was run on 10% polyacrylamide gel under non-reducing and reducing conditions by using 2-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie blue [11]. For Western blotting analysis, proteins were transferred from gels onto nitrocellulose membrane. Uncoupled active sites on the membrane's surface were blocked with 5% BSA, and incubated with the anti-TNF-α antibody followed by donkey anti-rabbit IgG antibody conjugated with peroxidase. After washes with PBS, the substrate was added for initialization of the color reaction [11].

2.8. Immunohistochemical study

Binding of purified FV/TNF-α onto formalin-fixed human colon cancer tissue sections was determined using a modification of the avidin–biotin–peroxidase complex (ABC) method as described in Ref. [11]. Briefly, human colon adenocarcinoma tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched by incubating the slides with 3% hydrogen peroxide in methanol for 15 min followed by three PBS rinses. The slides were incubated for 20 min in 10% normal rabbit serum to reduce any non-specific

staining and then incubated with FV/TNF-α (20 μg/ml) overnight at 4°C. All slides were washed three times in PBS and then incubated with biotinylated rabbit anti-TNF-α antibody (10 μg/ml) overnight at 4°C. After another three washes with PBS, the slides were incubated with the ABC reagent for 30 min. The peroxidase activity was developed with freshly prepared 0.06% 3′,3′-diaminobenzidine containing 0.1% hydrogen peroxide. Hematoxylin was used as a counterstain.

3. Results and discussion

3.1. Screening of the elution buffers

Twenty three candidates of various elution buffers were tested in the ELISA-elution assay. Fig. 1 showed the effects of different elution reagents on dissociation of bound FV/TNF-α from BSM. Elution buffers including the protein denaturants such as 8 *M* urea (A1 in Table 1), 6 *M* guanidine chloride (A2 in Table 1), very acidic pH (B1 and B2 in Table 1), very basic pH (B3 and B4 in Table 1), strong chaotropic ions (I^- and SCN^-) (D4, D5, E4 and E5 in Table 1) and high concentrations of the polarity-reducing agent ethylene glycol (70% or more) (F2 and F3 in Table 1) are very effective in disrupting the FV/TNF-α/TAG72 interaction. Their respective efficacies in dissociating FV/TNF-α from the BSM-coated plate are more than 75% compared to the standard FV/TNF-α binding curve (Fig. 1).

Magnesium chloride was much less effective in dissociating the FV/TNF-α/TAG72 complex, although it is usually effective at 2.5 *M* for purification of proteins [12]. Durkee et al. found that 3 *M* $MgCl_2$ was an efficient non-denaturing eluent which resulted in more than 90% enzyme protein recovery from their antibody-affinity column [13]. However, as shown in Fig. 1, the dissociation was only 10–15% at 1.3 *M* and 2.5 *M* $MgCl_2$ (C1 and C2 in Table 1). Even at 5 *M* $MgCl_2$ (C3 in Table 1) with high ionic strength and the strong chaotropic effect of Cl^- anions, the dissociation efficacy only reached 65%. Another elution buffer composed of a mixture of 3.0 *M* magnesium chloride and 25% ethylene glycol, pH

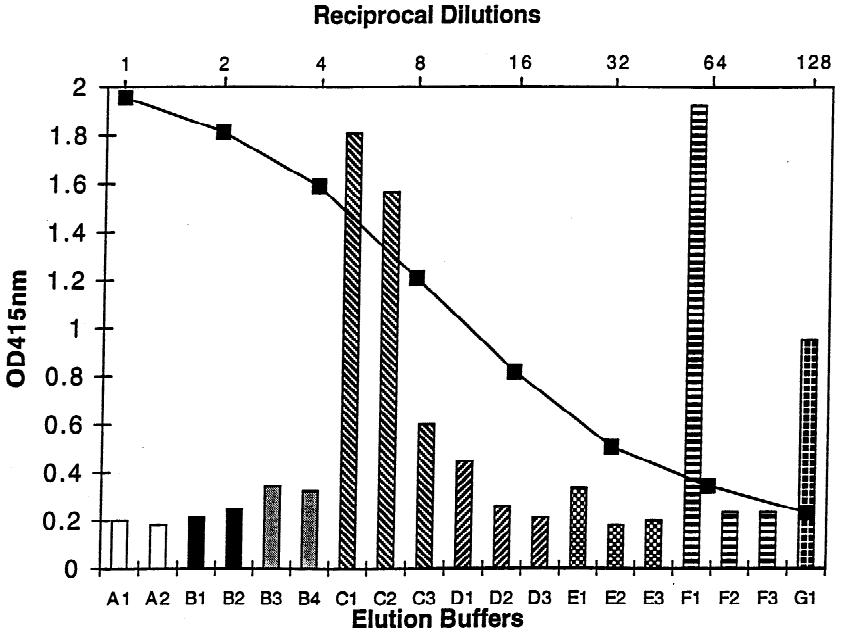

Fig. 1. Dissociation effect of elution buffers. Line: standard FV/TNF-α binding curve. The *E. coli* supernatant containing FV/TNF-α and its two-fold dilutions were added to BSM-coated wells. Bars: bound FV/TNF-α from the *E. coli* supernatant, which still remained on the plate after treatment with various elution buffers as determined by the ELISA-elution assay. Representative codes for various elution buffers are shown in Table 1. Standard deviations of each point are less than 5%.

7.2 (G1 in Table 1) had been claimed to be an effective eluent with respect to the total quantitative recovery of some proteins subjected to immunoaffinity purification [2]. However, as shown in Fig. 1, only about 50% of FV/TNF-α was ''released'' from the BSM-coated plate.

Since the non-covalent coating of BSM to the polystyrene microtiter plate is effected through hydrophobic and van der Waals forces [14], the use of elution buffers may strip off the bound BSM on the plate and interfere with the results obtained from the ELISA-elution assay. To rule out this possibility, we performed another ELISA assay to determine whether the coated BSM was removed from the plate by elution buffers. Twelve elution buffers including A1, B1, B4, C3, D3–5, E3–5, F3 and G1 (Table 1), which represent effective eluents in each group of elution buffers, were selected for further evaluation. As shown in Fig. 2, none of these 12 elution buffers stripped BSM off the plate, indicating that all of them are really effective in the disruption of the FV/TNF-α/TAG72 interaction. Our data are also consistent with a previous report which showed that the coating of a protein antigen to the polystyrene microtiter plate was so strong that desorption of coated antigen from the plate with 1.0 *M* NaCl, 6 *M* urea or 0.1% acetic acid, pH 3.0 for seven days removed 1.6% of coated antigen [15].

3.2. Denaturing effect of elution buffers

The denaturing effect of these 12 elution buffers on the fusion protein FV/TNF-α was evaluated in another ELISA. As shown in Fig. 3, reduction of FV/TNF-α-binding to the TAG72 epitope as a consequence of protein denaturation, varied depending on the type of elution buffers. The TAG72-binding activity of FV/TNF-α treated with the elution buffer (0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7) (B1 in Table 1) was completely lost, indicating that severe protein denaturation had occurred (Fig. 3A). Denaturants are usually the last choice for elution buffers since they have a strong detrimental effect on all proteins. Not surprisingly, the treatment of FV/TNF-α with 5 *M* urea caused almost 50% loss of the TAG72-binding activity (Fig. 3A). Although mag-

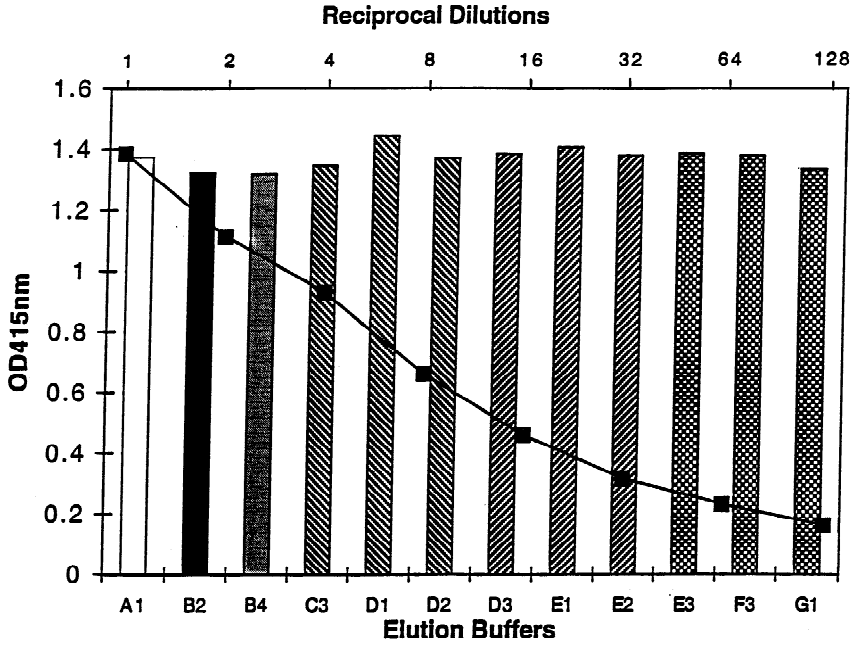

Fig. 2. Effect of elution buffers on removal of TAG72 from the plate. Line: standard TAG72-binding curve. Two hundred nanograms of BSM and its two-fold dilutions were coated onto the plate. Bars: bound BSM which remained on the plate was detected in ELISA. Representative codes for various elution buffers are shown in Table 1. Standard deviations of each point are less than 5%.

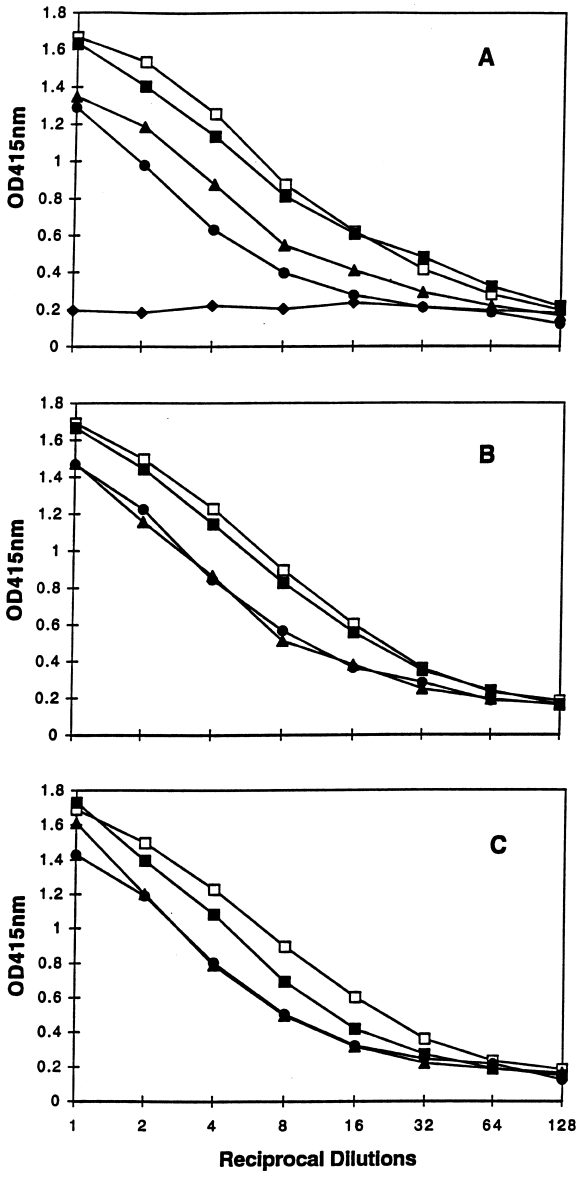

Fig. 3. Denaturing effect of different elution buffers. The *E. coli* supernatant containing FV/TNF-α was treated with different elution buffers including (A) no treatment control (□), 1.0 *M* ammonia solution, pH 11.5 (■), 5.0 *M* urea (▲), 5.0 *M* magnesium chloride (●), 0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7 (♦); (B) no treatment control (□), 1.3 *M* potassium iodide (■), 2.5 *M* potassium iodide (▲), 5.0 *M* potassium iodide (●); (C) no treatment control (□), 0.9 *M* potassium thiocyanate (■), 1.8 *M* potassium thiocyanate (▲), and 3.5 *M* potassium thiocyanate (●).

nesium chloride (C1–3 in Table 1) had limited ability in the dissociation of the FV/TNF-α/TAG72 interaction, it surprisingly decreased the TAG72-binding activity of FV/TNF-α after treatment of the FV/TNF-α with 5 *M* magnesium chloride by nearly 75% (C3 in Table 1).

Three elution buffers including 1 *M* ammonia solution, 1.3 *M* potassium iodide and 0.9 *M* potassium thiocyanate (B4, D3 and E3 in Table 1) were found to have a minimal denaturing effect. The TAG72-binding activity of FV/TNF-α treated with these three elution buffers were fairly comparable to the untreated control sample (Fig. 3A–C). These three elution buffers are thus suitable for purification of FV/TNF-α by using the TAG72-affinity column. Since the eluted FV/TNF-α has to be extensively dialyzed against PBS before use, we therefore favored the use of 1 *M* ammonia solution because, as a volatile buffer system, it can be easily evaporated during the lyophilizing process and did not effect dialysis.

The *E. coli* culture supernatant containing FV/TNF-α was applied to the TAG72 affinity column and the fractions containing FV/TNF-α was eluted with 1 *M* ammonia solution, pH 11.5. To examine its purity, the eluted protein sample was further subjected to SDS–PAGE analysis. After electrophoresis, the gel was stained with Coomassie blue. As shown in Fig. 4A and B, the fusion protein FV/TNF-α displayed a single band of M_r 43 000 under both non-reducing and reducing conditions, indicating the existence of a single-chain fusion protein of M_r 43 000. To confirm the presence of TNF-α moiety, Western blotting analysis was performed. As shown in Fig. 4C and D, the rabbit anti-TNF-α antibody detected the bands at their respective locations of molecular mass of 43 000, indicating the presence of the TNF-α moiety in the fusion protein. The total yield of FV/TNF-α by the TAG72-affinity chromatography using 1 *M* ammonia solution, pH 11.5 as the eluent was estimated to be 1.5 mg from 1 l of the *E. coli* supernatant containing FV/TNF-α compared with only 0.005 mg when using 0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7 for protein elution. To confirm the TAG72-binding activity of purified FV/TNF-α, we conducted an immunohistochemical study by using human colonic adenocarcinoma tissue sections. The binding of FV/TNF-α was confirmed by using

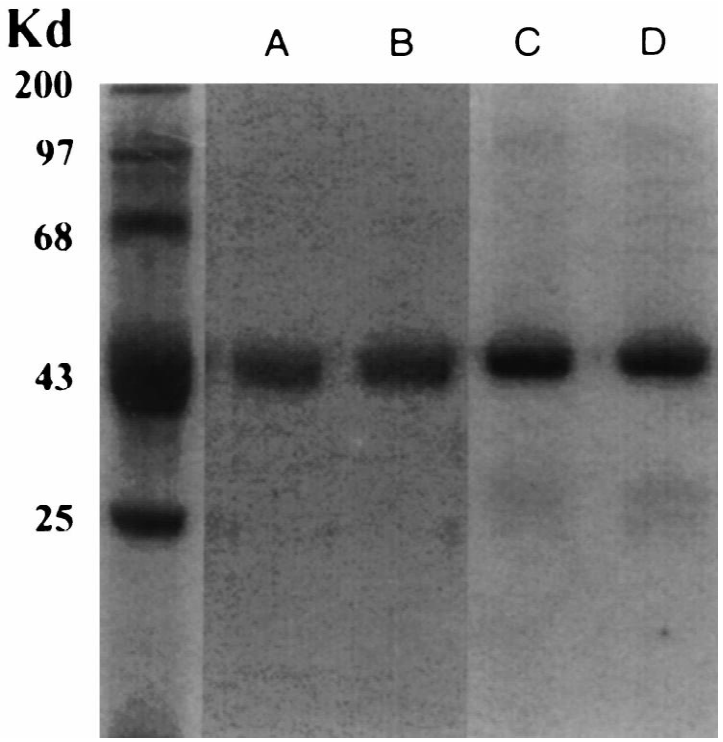

Fig. 4. SDS–PAGE and Western blotting analysis of purified FV/TNF-α. Protein samples were run on polyacrylamide gels (10%) under non-reducing (A) and reducing conditions by using 2-mercaptoethanol (B). After electrophoresis, the gel was stained with Coomassie blue. In Western blotting analysis, proteins which were run on gels under non-reducing and reducing conditions were transferred onto the nitrocellulose membrane (C) and (D). TNF-α moiety was detected by rabbit anti-TNF-α antibody. Left side: molecular mass markers.

biotinylated rabbit anti-TNF-α antibody followed by peroxidase conjugated avidin. As shown in Fig. 5, FV/TNF-α showed specific interactions with complementary structure, i.e., binding sites, located on colonic adenocarcinomatous cells. This presumably involves similar binding sites as, e.g., the TAG72 epitope on the surface on BSM type I-S. This positivity predominantly involved the some suprabasal aspects of the tumor cells. In contrast, the normal colonic mucosa showed no positive effect.

In summary, the ELISA-elution assay was developed to screen a large variety of elution buffers with respect to their efficiency for disrupting FV/TNF-α/TAG72 interaction. Ammonia solution (1 *M*, pH 11.5) was finally selected because of its effective dissociation of more than 75% of the FV/TNF-α/TAG72 complex, its minimal protein denaturing effect on FV/TNF-α and its easy removal from the eluted samples containing the purified FV/TNF-α. The total yield of purified FV/TNF-α using this elution buffer is 300-fold higher than that using the common elution buffer 0.1 *M* glycine, 0.5 *M* NaCl, pH 2.7. Our study underlines that the ELISA-elution assay will be useful in the selection of suitable elution buffers for affinity chromatography.

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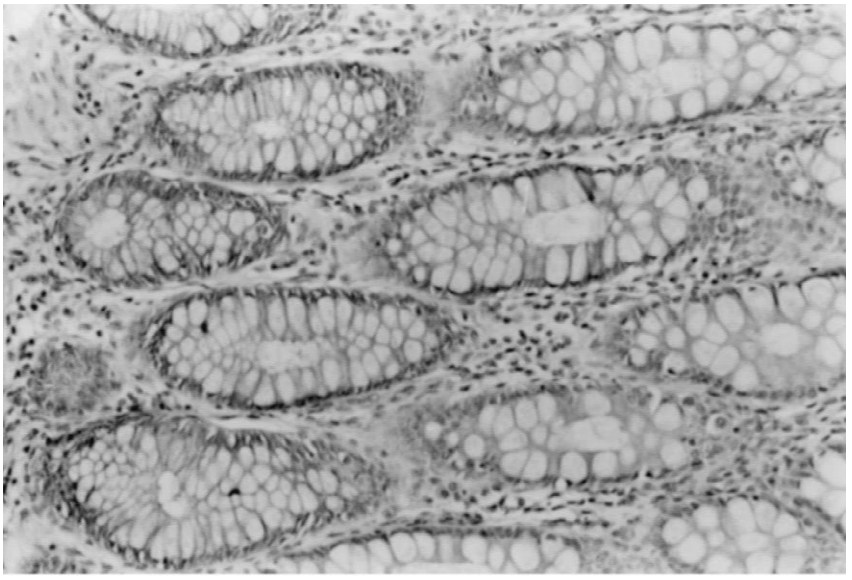
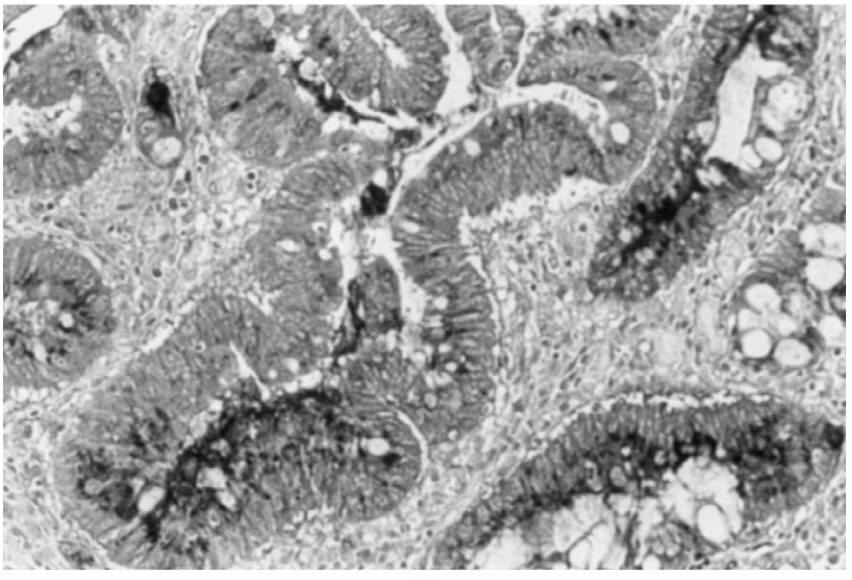


(a)



(b)

Fig. 5. Immunohistochemical study of formalin-fixed paraffin-embedded human colonic adenocarcinoma using FV/TNF-α and biotinylated rabbit anti-TNF-α antibody. (a) The normal colonic mucosa shows no binding of FV/TNF-α at all. (b) Note the focal areas of FV/TNF-α binding on the suprabasal and/or luminal aspects of the colonic adenocarcinomatous cells.

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