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Journal of Chromatography A, 679 (1994) 190–194

JOURNAL OF CHROMATOGRAPHY A

Short communication

Antibody against branched epitope as an affinity ligand to separate the parent protein

Z.J. Yao[a,*], M.C.C. Kao[a], K.C. Loh[a], M.C.M. Chung[a,b]

[a]*Bioprocessing Technology Unit, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Singapore*
[b]*Department of Biochemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Singapore*

First received 30 March 1994; revised manuscript received 9 June 1994

Abstract

A peptide that contained one of the continuous epitopes of recombinant human lymphotoxin (rhLT) (amino acid residues 139–154) has been located by epitope mapping. The branched form of this peptide was synthesized by the multiple antigen peptide procedure with an octameric branched resin and was subsequently used to elicit anti-epitope antibody in rabbits. The resulting anti-epitope was then used as an immunoaffinity ligand in affinity chromatography to purify the parent protein, rhLT, from the host cell lysate directly.

It is suggested that this approach would be a general way to create novel biospecific ligands for affinity separations.

1. Introduction

Immunopurification is one of most selective and powerful methods of protein purification [1]. Antibodies, for example, can readily distinguish between very similar antigens and therefore can overcome many of the separation problems that no other method can resolve. Both polyclonal and monoclonal antibodies can be used as the affinity ligand for this method of purification, but both have their unique advantages and limitations.

Polyclonal antibodies can be conveniently produced by injecting a purified antigen into a suitable animal and then harvesting the antibodies after 1–2 months time. However, since the resolution of separation is based on the specificity of an antibody, which in turn is mainly dependent on the purity of an antigen, an elaborate and often time-consuming purification protocol for the antigen usually has to be developed in advance. On the other hand, monoclonal antibodies are more specific in their interaction as they can recognize the epitope of a protein, but elicitation of monoclonal antibodies needs additional expertise and facilities for cell fusion. Therefore, it is thought that when the epitope(s) of an antigen is/are known (e.g., through epitope mapping), anti-epitope antibodies would be useful alternative affinity ligands for separation.

Several continuous epitopes of recombinant human lymphotoxin (rhLT or TNF-β) have been located and their topographies studied in our laboratory [2]. In this communication we wish to report on a branched epitope-carrying peptide that has been used as immunogen to elicit anti-

* Corresponding author.

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SSDI 0021-9673(94)00555-N

epitope antibody; the resulting antibody subsequently worked as an affinity ligand of chromatography to purify the parent protein (rhLT) efficiently. Because a branched peptide can be chemically synthesized with an automatic peptide synthesizer and its immunogenic property was reported to be stronger than the linear peptide, the branched peptide might be a common way to produce anti-epitope antibodies that exhibit similar specificity as a monoclonal antibodies, but which can be produced as conveniently as for a polyclonal antibody.

2. Materials and methods

2.1. Materials

Escherichia coli cell lysate containing rhLT was obtained from recombinant HB101 host cells [3]. Purified rhLT, which was used as a standard in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and for coating enzyme-linked immunosorbent assay (ELISA) plates, was purified from this cell lysate [4].

Freund's adjuvants and keyhole limpet hemocyanin were purchased from Sigma. Glutaraldehyde was obtained from Fluka. CNBr-activated Sepharose and SDS-PAGE molecular markers were from Pharmacia. Nitrocellulose membrane was purchased from Schleicher & Schuell while ELISA plates were from Nunc. Other chemicals and reagents not specifically mentioned were obtained from standard commercial sources.

2.2. Methods

Peptide synthesis

Peptides were synthesized with an ABI 431A peptide synthesizer using 9-fluorenylmethoxycarbonyl (FMOC) chemistry. A FMOC 8-branched multiple antigen peptide (MAP) [5] resin was used to synthesize the peptide based on the recommended procedure of the manufacturer [6]. Amino acids and other synthesis chemicals were purchased from Applied Biosystems (Foster City, USA).

Conjugation of peptide

The epitope-carrying peptide was coupled to hemocyanin using a two-step glutaraldehyde method [7].

Immunization

Emulsion solutions were prepared by mixing equal volumes of complete Freund's adjuvant with either branched peptide, peptide conjugated to hemocyanin or pure peptide separately. Two to three rabbits were used for immunization in each group. Complete Freund's adjuvant was used in the first injection, whereas incomplete adjuvant was used in subsequent booster injections. Rabbits were injected subcutaneously (at multiple points in the back region) at a dose of 0.5 mg peptide per animal. Booster injections were carried out at 2, 4 and 6 weeks after the first injection.

A polyclonal anti-rhLT antibody was similarly elicited by using purified rhLT as the immunogen.

ELISA

An indirect antibody ELISA method was used to measure the specificity of antibodies to rhLT. ELISA plates (96 wells) were first coated with purified rhLT (in 0.05 *M* carbonate–hydrogencarbonate buffer, pH 9.5) at 1 μg/well, followed by blocking with 1% (w/v) albumin. Antibodies diluted in 5% (w/v) skimmed milk solution were added to the wells and incubated overnight at 4°C, washing four times with washing buffer (0.05% Tween 20 in phosphate buffered saline, pH 7.4), then reacted with anti-rabbit immunoglobulin–horseradish peroxidase conjugate for 1 h at 37°C. Finally, after washing phenylenediamine dihydrochloride (OPD) was added as substrate and the absorbance was measured at 492 nm.

Affinity chromatography

Sera were bled on the 68th day from rabbits immunized by the branched peptide. The immunoglobulin was purified from pooled sera by

DEAE-cellulose ion-exchange chromatography [8]. Affinity separation was performed on a column prepared by coupling the purified immunoglobulin to CNBr-activated Sepharose (8 × 1 cm). The column was pre-equilibrated with 10 m*M* Tris buffer, pH 7.5. After removing the unbound fraction, the bound fraction was eluted with 100 m*M* triethylamine, pH 11.5. These collected fractions were neutralized with 0.5 *M* HCl immediately.

Protein estimation

Protein estimation was performed according to the method of Bradford [9] using the reagents purchased from Bio-Rad Labs.

SDS-PAGE and Western blotting

SDS-PAGE was carried out based on Laemmli's procedure [10] and the separated proteins were visualized by Coomassie Brilliant Blue or silver staining. Western blotting was performed at room temperature and 30 V overnight using a mini-transblot cell (Bio-Rad Labs.). The blotting membrane was blocked with 5% (w/v) skimmed milk powder before being incubated with anti-rhLT antibody overnight at 4°C. This was followed by incubation with anti-rabbit antibody–horseradish peroxidase conjugate (Silenus Labs.) for 1 h at room temperature. The membrane was finally developed with 4-chloro-1-naphthol.

3. Results and discussion

3.1. Multiple antigen peptide of human lymphotoxin epitope

Peptide mapping studies of rhLT showed that the peptide with the sequence FQLTQGDQLSTHTDGI (residues 139–154, molecular mass 1761) displayed the strongest antigenicity when cross-reacted with anti-hLT antibodies among the rhLT fragments investigated. This peptide was subsequently shown to contain an epitope, FQLTQGDQL, residues 139–147 of this cytokine [2]. This 16 amino acid residue peptide FQLTQGDQLSTHTDGI was synthesized as an 8-branched MAP to elicit anti-peptide antibodies. The branched peptide displayed a single broad peak on reversed-phase HPLC and gave the expected amino acid composition (data not shown). SDS-PAGE analysis showed that it had a higher apparent molecular mass of approximately 26 000 (Fig. 1) as compared to the expected value of 14 100

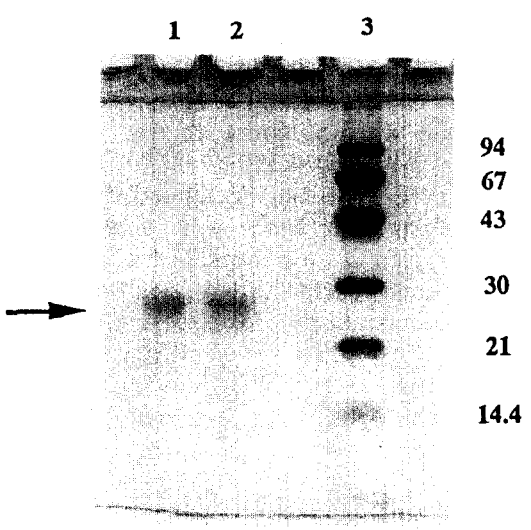

Fig. 1. The apparent molecular mass of branched epitope on SDS-PAGE. Lanes: 1, 2 = branched epitope; 3 = molecular mass markers (M_r indicated in kilodalton).

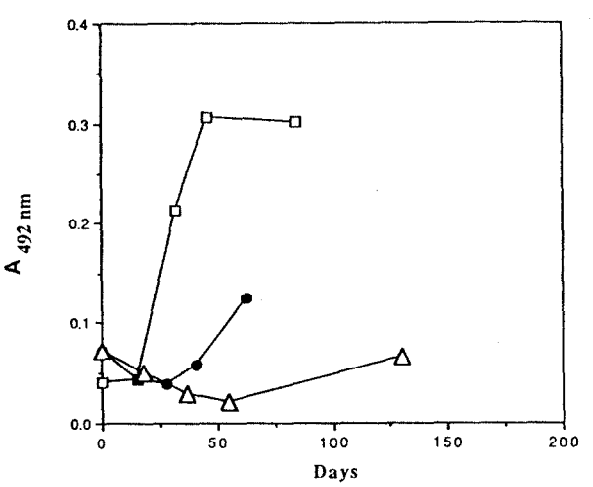

Fig. 2. Specific binding of antibodies to parent protein, rhLT. □ = antibody vs. branched epitope; ● = antibody vs. conjugate of epitope-hemocyanin; △ = antibody vs. the linear peptide-carrying peptide.

(FQLTQGDQLSTHTDGI × 8 = 1761 × 8). This could be due to the extensive branching of the MAP which may have in some way retarded the mobility of the peptide and/or reduced the expected stoichiometric binding of SDS (1.4 g SDS/g protein) to the peptide.

3.2. Anti-peptide titre and specificity of rabbit antibody

For comparison, the immunogenicity of the branched peptide was compared with that of two other immunogenic methods: peptide coupled to hemocyanin and the linear peptide itself being used as the immunogens. The results showed that significant antibody titres were obtained in the branched peptide and peptide–hemocynin groups after 6–8 weeks, whereas there was little response being observed in the linear peptide group after the same period of time.

ELISA was carried out to evaluate the specific binding of the antibodies to rhLT. Although both antibodies from the branched peptide and the peptide–hemocynin conjugate displayed specific binding to rhLT, the antibody from the branched one exhibited a stronger response (Fig.

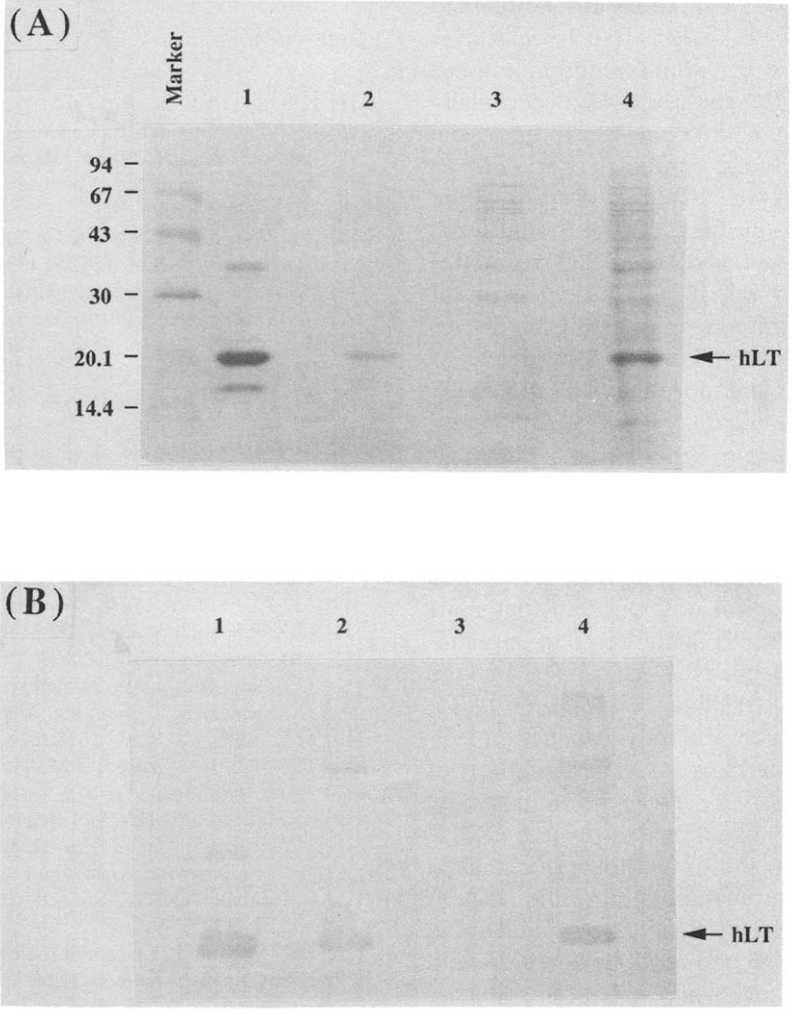

Fig. 3. SDS-PAGE (A) and Western blot (B) of the fractions derived from affinity chromatography. Lanes: 1 = purified rhLT (as standard); 2 = bound fraction; 3 = unbound fraction; 4 = cell lysate before separation. M_r indicated in kilodalton.

2). These results seemed to confirm that branched epitope are better immunogens in producing anti-peptide antibodies [11,12].

3.3. Immunoaffinity chromatography

When purified rhLT was applied to the affinity gel column (prepared by coupling purified immunoglobulin against the branched peptide to activated Sepharose 4B), the parent protein, rhLT, was adsorbed to the column quantitatively and the capacity of the affinity gel was proportional to the amount of antibody coupled to the gel matrices (data not shown).

When the cell lysate containing rhLT obtained from recombinant *E. coli* HB101 cells was loaded directly onto the affinity column without any pretreatment, the rhLT was also specifically retained. The bound fraction could be easily recovered by applying elution buffer. Fig. 3 shows the SDS-PAGE (A) and corresponding Western blot (B) profiles of the bound and unbound fractions obtained from this chromatographic separation step. It clearly demonstrated that rhLT was separated from most of the *E. coli* proteins in a single step. These separations were highly reproducible and more than 100 runs were performed in the same column without any decrease in efficiency.

The results reported here demonstrated that a protein epitope synthesized as a branched peptide is a valuable alternative for eliciting anti-peptide antibodies as affinity ligands for protein purification. Although it was reported that some synthetic linear peptides might be good immunogens [13], other peptides, such as the peptide used in this study (residues 139–154 of hLT), was unable to induce antibody production efficiently. Some procedures, such as coupling of the pure peptide to a carrier protein or by peptide cyclization, were used to overcome this problem [14], but these methods often need additional chemical manipulations and exhibit variable efficiency on a case to case basis. On the other hand, a branched epitope peptide can enhance the immunogenicity without introducing extra foreign sequences. Moreover, the branched peptide can be synthesized automatically on a peptide synthesizer.

Recently several more rapid epitope mapping methods have been developed [15]. Some of these methods are based on the so-called random peptide library approach in which epitope mapping is performed without any prior knowledge of the protein sequence [16,17]. As the branched epitope procedure can be readily linked with epitope mapping to produce biospecific ligands for affinity chromatography, this may lead to a wider application of anti-epitope antibodies for protein purification in the future.

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