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A ten-residue fragment of an antibody (mini-antibody) directed against lysozyme as ligand in immunoaffinity chromatography

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ABSTRACT

The interaction between an antibody molecule and a protein antigen is an example of "natural" protein modelling. Amino acids of the antigen-binding site consisting of three hypervariable segments (L1, L2, L3) of the light (L) and three (H1, H2, H3) of the heavy (H) chain of an antibody molecule interact with amino acids present in an epitope of a protein. A ten-residue peptide was synthesized with an amino acid sequence analogous to the hypervariable L3 segment of a monoclonal antibody directed against lysozyme. The peptide was immobilized on CH-Sepharose 4B and the affinity adsorbent was used to purify lysozyme added to a detergent extract of insect cells infected with a recombinant baculovirus. This methodology may also be applicable to other antigen–antibody combinations, in immunoaffinity chromatography for selective purification of a protein or in an immunosensor for detection of a protein.

INTRODUCTION

Multiple non-covalent forces, hydrogen bonding, electrostatic, hydrophobic and van der Waals forces play a role in the binding between a protein antigen and an antibody directed against this protein. The antigen is bound by the antigen-binding site of an antibody, which consists of three hypervariable segments of the light chain (L1, L2, L3) and three of the heavy chain (H1, H2, H3) (Fig. 1).

Antibodies can be raised against a fragment of a protein (a peptide) and these antibodies may react with the intact protein [1]. Apparently, the conformation of the peptide is, at least partly, similar to that of the intact protein. Recently, the tertiary structure of a complex of a nineteen-residue peptide and a monoclonal antibody

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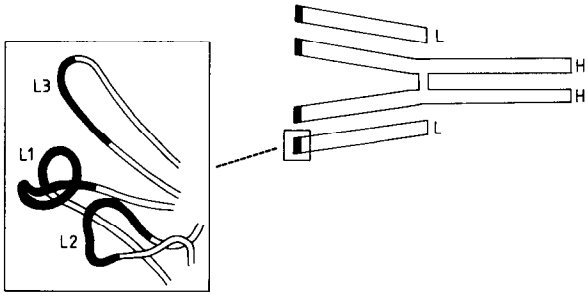

Fig. 1. Schematic representation of an immunoglobulin G molecule. One antigen-binding site (indicated in black) is composed of three hypervariable polypeptide segments of the light chain (L) and three of the heavy chain (H). Inset, spatial location of hypervariable segments (L1, L2, L3) in the L chain.

directed against this peptide was determined [2]. The interaction between the peptide and, in this instance, five of the hypervariable segments is governed by the above-mentioned non-covalent forces. We assume that selected fragments of an antibody may bind a protein antigen by the same forces. In an earlier study [3], it was shown that a synthetic thirteen-residue peptide with an amino acid sequence analogous to that of the H2 segment of a monoclonal antibody (Gloop2) raised against loop peptide (residues 57–83) of lysozyme [4–6] was able to bind lysozyme. In this study, the binding properties of a synthetic ten-residue peptide from the L3 segment of this anti-lysozyme monoclonal antibody were investigated.

EXPERIMENTAL

Peptide selection, synthesis and characterization

Using the modelling studies of complexes of monoclonal antibodies and lysozyme [5,6], a ten-residue peptide of the L3 segment was selected for synthesis, *viz.*, Tyr–Leu–Ser–Tyr–Pro–Leu–Thr–Phe–Gly–Ala. The peptide was synthesized by the semi-automatic solid-phase method with α-amino groups protected with the 9-fluorenylmethyloxycarbonyl (Fmoc) group [7,8].

The purity of the peptide was verified by reversed-phase (RP)-high-performance liquid chromatography (HPLC), by amino acid analysis and by determination of the N-terminal amino acid by dansylation [9].

Affinity chromatography

The immunoaffinity adsorbent was prepared by coupling of the synthetic L3 peptide (2 mg) to 0.5 g of activated CH-Sepharose 4B obtained from Pharmacia–LKB (Uppsala, Sweden) in 0.1 *M* $NaHCO_3$–Na_2CO_3 (pH 8.2) containing 0.5 *M* sodium chloride according to the instructions of the manufacturer. The percentage coupling was determined by RP-HPLC of the peptide solution before coupling and the eluate after coupling. More than 99% of the peptide was coupled to the activated column material, resulting in 1.8 μmol of peptide per gram of dry gel. Excess active groups were blocked by washing with 0.1 *M* Tris–HCl (pH 8.0). A control column was prepared by blocking active groups with 0.1 *M* Tris–HCl (pH 8.0). Affinity

chromatography was carried out at room temperature at a flow-rate of 10 ml/h during application of samples and 20 ml/h during chromatography. Columns were eluted with 0.05 *M* sodium thiocyanate in 20 m*M* Tris–HCl (pH 7.4), followed by 1 *M* sodium thiocyanate in the same buffer. The absorbance was measured at 280 nm.

Complex mixture of proteins

Insect cells (sf21) were infected with recombinant baculovirus (AcgD) expressing glycoprotein D of herpes simplex virus. Cells were harvested 50 h after infection and washed twice with Hanks balanced salt solution. After resuspending the cell pellet (10^7 cells per ml) in 10 m*M* Tris–HCl (pH 7.4), containing 2 m*M* phenylmethylsulphonyl fluoride (PMSF) and 1 m*M* Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), the same volume of a buffer consisting of 4% decyl polyethylene glycol 300 (Kwant–Hoog Vacolie Recycling and Synthese, Bedum, Netherlands), 10 m*M* Tris–HCl (pH 7.4), 2 m*M* PMSF and 1 m*M* TLCK was added and the mixture incubated for 30 min at 0°C. Cell debris was removed by low-speed centrifugation, followed by ultracentrifugation at 100 000 *g* for 1 h. The supernatant containing the extracted proteins was stored in aliquots at −80°C until used. Prior to immunoaffinity chromatography, lysozyme (250 μg in 100 μl of water) was added to 500 μl of an extract of insect cells containing 1.2 mg protein per ml.

RP-HPLC

RP-HPLC was performed with a Pharmacia-LKB system consisting of a Model 2150 HPLC pump, Model 2152 LC controller, Model 11300 Ultrograd mixer driver, a Rheodyne Model 7125 injector, Model 2151 variable-wavelength monitor and a Model 2210 recorder. The column (25 × 4.6 mm I.D.) contained Nucleosil 10 C_{18} (Macherey, Nagel, & Co., Düren, Germany). Proteins and peptides were eluted with a gradient from 10% acetonitrile in 0.1% trifluoroacetic acid (TFA) to 40% acetonitrile in 0.09% TFA (30 min) and then 66% acetonitrile in 0.09% TFA (5 min). The absorbance was monitored at 214 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of 50 μl of the eluate fractions were analysed on 13.5% SDS polyacryl amide gels [10]. Polypeptide bands were revealed with a silver-staining method [11].

RESULTS AND DISCUSSION

It was shown previously [3] that an immobilized synthetic thirteen-residue peptide from the antigen-binding H2 region of a monoclonal antibody (raised against lysozyme loop peptide residues 57–83 and also reactive with intact lyzozyme) could be used to purify lysozyme from a mixture of proteins. Three other unrelated synthetic peptides with charges ranging −6 to +2 did not bind lysozyme [3]. In the present study, another, smaller part of the antigen-binding site of the same monoclonal antibody was investigated. A ten-residue peptide with an amino acid sequence analogous to that of the L3 segment was synthesized.

In Fig. 2a a space-filling model of the antigen binding site is shown in which the surface residues present in the thirteen-residue H2 peptide and the ten-residue L3 peptide are indicated [6]. In addition, the residues of the epitope on lysozyme with

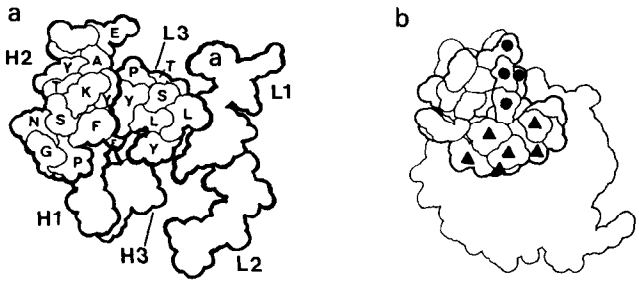

Fig. 2. (a) All-atom space-filling representation of the antigen-binding site perpendicular to the view in Fig. 1 of monoclonal antibody Gloop2 directed against hen egg-white lysozyme. Only the surface amino acid residues present in the synthetic versions of H2 and L3 are indicated by the one-letter code for amino acids. From ref. 6, by permission of Oxford University Press. (b) Space-filling model of lysozyme in which the epitope interaction with Gloop2 is indicated. (▲) Residues interacting with H2; (●) residues interacting with L3. From ref. 5, by permission of Oxford University Press.

which H2 and L3 interact are shown [5] (Fig. 2b). As the L3 peptide does not contain a lysine it could be attached to activated gels via its N-terminal amino acid. However, initial experiments showed that coupling of this peptide to tresyl-activated Sepharose resulted in material that could not bind lysozyme. One of the reasons might be the lack of spatial freedom owing to the close proximity of the gel matrix. This might limit the number of conformations of the peptide and hence the possibility that the peptide may exist in the optimum conformation to interact with the relatively large protein antigen. Therefore, the peptide was coupled to activated CH-Sepharose 4B which contains a six-carbon atom spacer between the activated group and the gel matrix. This material was used for affinity chromatography.

Fig. 3 shows that lysozyme was bound to the column material with the ten-residue peptide as ligand whereas it was not bound by a control column deactivated

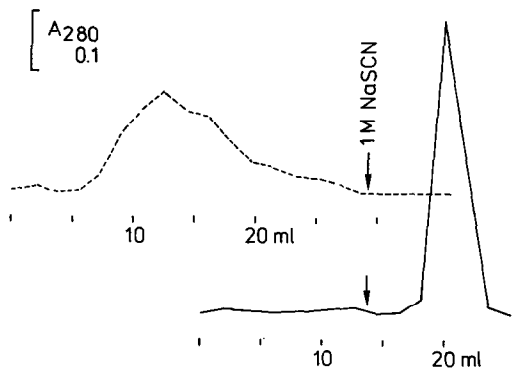

Fig. 3. Affinity chromatography of lysozyme. A ten-residue peptide from the L3 segment of a monoclonal antibody against lysozyme was coupled to activated CH Sepharose 4B. A control adsorbent was prepared by washing with 0.1 *M* Tris–HCl (pH 8.0). Lysozyme (1.6 mg) was applied to each adsorbent. The columns were eluted with 0.05 *M* sodium thiocyanate in 20 m*M* Tris–HCl (pH 7.4). The arrow indicates the start of elution with 1 *M* sodium thiocyanate in the same buffer. The flow-rate was 10 ml/h during application of the sample and 20 ml/h during chromatography. The absorbance was measured at 280 nm. Solid line, anti-lysozyme column; dashed line, control column.

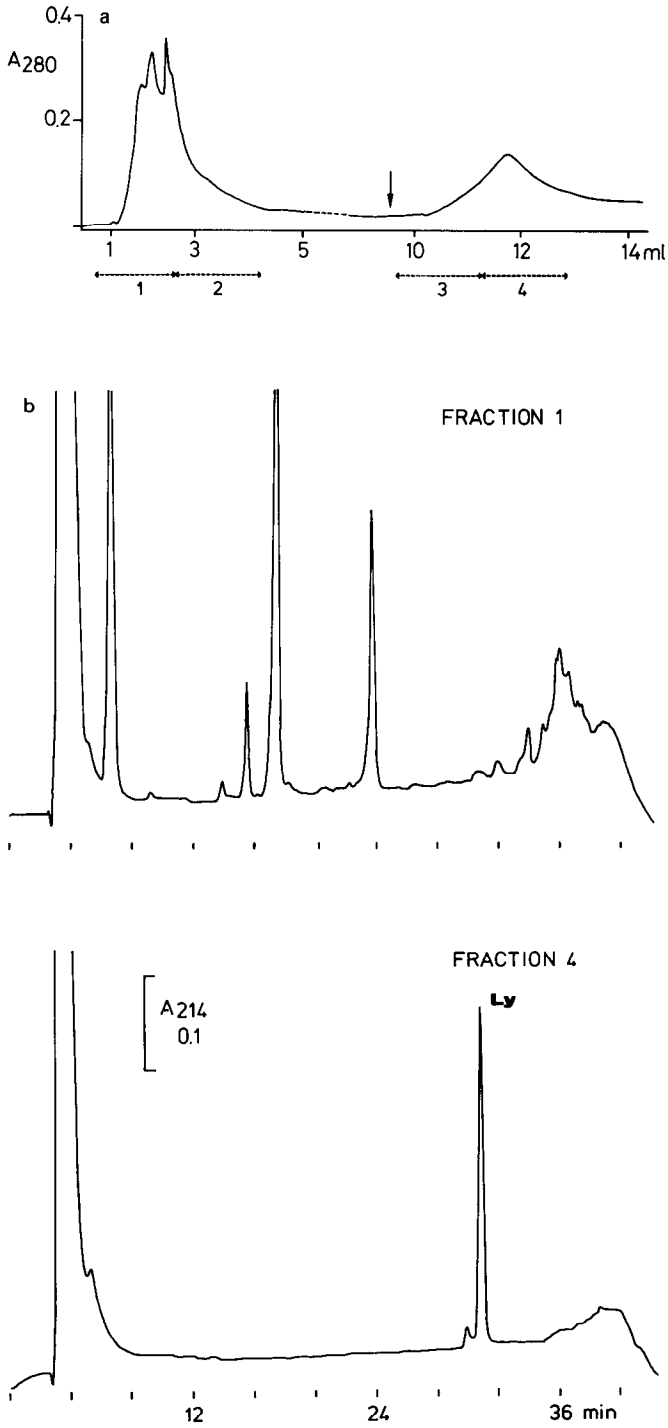

Fig. 4.

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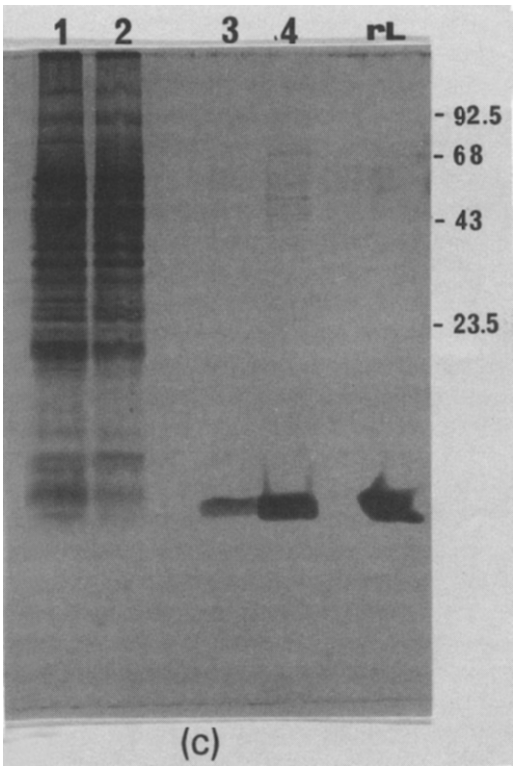

Fig. 4. (a) Affinity chromatography with the anti-lysozyme adsorbent. A 600-μl volume of a detergent extract of insect cells infected with a recombinant baculovirus (1.2 mg protein per ml) containing 250 μg of lysozyme was applied to the column. Elution conditions as in Fig. 3. Aliquots of the indicated fractions were analysed by (b) RP-HPLC (300-and 100-μl of fractions 1 and 4, respectively); the large peaks at 4 min contain sodium thiocyanate and the small peak in front of the lysozyme peak is also present in a reference sample; Ly, lysozyme; (c) SDS-PAGE (50-μl of fractions 1–4); rL, reference solution of lysozyme. The molecular weights of reference proteins are indicated in kilodaltons.

with Tris–HCl. Binding constants were not determined but further affinity chromatographic experiments showed that lysozyme was eluted slowly from the anti-lysozyme column between 0.1 and 0.2 *M* sodium thiocyanate.

It was then investigated whether the binding was sufficiently selective to purify lysozyme from a complex mixture of proteins. Lysozyme (250 μg) was added to a detergent extract of insect cells infected with a recombinant baculovirus. This extract contains 1.2 mg protein per ml, 1.7% of the non-ionic detergent decyl polyethylene glycol and protease inhibitors. This mixture was applied to the anti-lysozyme column and the elution pattern is shown in Fig. 4a. Fractions were analysed by RP-HPLC and SDS-PAGE (Fig. 4b and c). This showed that the proteins present in the original cell extract were almost exclusively present in fractions 1 and 2 whereas lysozyme was absent and that a minor amount of these proteins (molecular weights of 40–70 kilodalton) and lysozyme were present in the peak eluted by 1 *M* sodium thiocyanate.

The response of the immune system on contact with a foreign compound, *e.g.*, a protein, is the production of antibodies. The interaction between an antibody molecule and a protein antigen is an example of 'natural' protein modelling. Binding constants are often high and in immunoaffinity chromatography severe conditions are often required for elution of proteins. As a consequence, the native structure of both antibody and protein may be affected. By reducing the immunoligand to its smallest possible size, binding constants will be considerably lower and thus elution of proteins may occur under relatively mild conditions. In general, elution may probably be achieved at concentrations less than in 1 *M* sodium isothiocyanate used in our study, but under such conditions the peak will be broader. Although peptides are more temperature stable than proteins, they are generally more susceptible to proteolytic degradation. Therefore, depending on the sample, the addition of protease inhibitors or a lower temperature (4°C) would be advisable during immunoaffinity chromatography.

So far only anti-lysozyme peptides have been studied and the selection of peptides was facilitated by the availability of a predicted model of the tertiary structure of an anti-lysozyme monoclonal antibody [6]. We suggest that mini-antibodies (antigen-binding fragments of an antibody) may be constructed either by organic synthesis or by recombinant DNA techniques [12–14]. Tailor-made mini-antibodies might be produced, *i.e.*, against a particular protein according to the following scenario. In hybridoma cells the concentration of immunoglobulin mRNA is relatively high. After hybridization the the RNA of the L or H chain with an oligonucleotide complementary to the constant region, elongation will be possible in the direction of the variable region, *i.e.*, synthesis of cDNA, which then can be sequenced. The amino acid sequence can be deduced from the DNA sequence and the hypervariable segments can be located. In the absence of tertiary structure information, it is then necessary to investigate the binding properties of all six antigen-binding segments. The optimum fragment or combination of fragments can then be used in immunoaffinity chromatography for selective purification of a protein or in immunosensors for detection of a protein.

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