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Synthetic antibody fragment as ligand in immunoaffinity chromatography

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ABSTRACT

The possibility that a fragment of an antibody molecule may interact with a protein antigen was tested by studying the binding properties of a thirteen-residue synthetic peptide with an amino acid sequence similar to part of a hypervariable segment of a monoclonal antibody directed against lysozyme. Affinity adsorbents were prepared with this peptide and with non-related peptides as ligand. Non-specific interactions could be abolished by washing the column with 0.05 *M* sodium thiocyanate in 20 m*M* Tris–HCl (pH 7.4). Lysozyme was only bound to the antilysozyme adsorbent and could be eluted with 1 *M* sodium thiocyanate. The results show that immunoaffinity chromatography with synthetic peptide ligands which mimic the antigen-binding site may be a useful tool in the selective purification of proteins.

INTRODUCTION

One of the most selective biological interactions is that between antigen and antibody. Multiple non-covalent forces, hydrogen bonding, electrostatic, hydrophobic and Van der Waals forces are responsible for the antigen antibody reaction.

The aim of this study was to exploit the recognition potential of an antibody for the purification of a particular protein by using a peptide ligand which mimics the antigen-binding site of a monoclonal antibody molecule directed against this protein. Immunoglobulin G molecules are large (M_r = 150 000). A relatively small section of the molecule (see Fig. 1a), consisting of three hypervariable segments of the L chain

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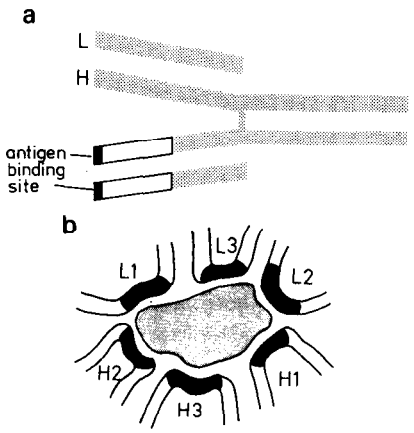

Fig. 1. (a) Schematic representation of an immunoglobulin G molecule. Open blocks indicate the variable regions of the heavy (H) and light (L) chains. One antigen-binding site is composed of three hypervariable polypeptide segments (indicated in black) of the L chain and three of the H chain [see (b)]. (b) Schematic representation of the antigen-binding site [perpendicular to the view in (a)]. The hypervariable segments L1, L2, L3, H1, H2 and H3 are indicated in black. The antigenic determinant is shaded.

and three of the H chain (Fig. 1b), binds to the antigen, often with high affinity (K_{diss} = 10^{-8}–10^{-12} *M*). For this reason, severe conditions are often required for elution of the protein antigen in conventional immunoaffinity chromatography. Other parts of the immunoglobulin molecule may show aspecific interactions with contaminating proteins, which may interfere with the purification of the protein antigen. When the antibody molecule is reduced to its smallest possible biologically active size, *i.e.*, still showing antigen-binding properties, the aforementioned aspecific interactions will no longer occur. Binding will be less strong, especially when only part of the antigen-binding site is used as a ligand. However, this may also be favourable as the protein can then be eluted under relatively mild conditions.

To test this hypothesis, we used a model of an antigen–antibody complex, *i.e.*, a lysozyme–anti-lysozyme complex[1–3]. The antibody segment with the largest number of contact residues was selected for synthesis. This synthetic thirteen-residue anti-lysozyme peptide was immobilized and used to purify lysozyme from a mixture of proteins.

EXPERIMENTAL

Peptide selection and synthesis

An anti-lysozyme (anti-LY) peptide was selected (Table I) using the modelling studies of complexes of monoclonal antibodies and lysozyme[2,3]. A peptide with as many contact residues as possible in a linear sequence was synthesized by the solid-phase methodology[4]. Boc-Cys-3-nitro-2-pyridinesulphenyl-OH [Boc-Cys-(NPys)-OH] was coupled as an N-terminal residue to the resin-linked peptide to allow specific coupling to its N-terminus[5]. After deprotection and cleavage from the resin, the NPys-cysteinyl peptide (6 μmol = 10.4 mg) was added to thiopropyl-Sepharose 6B

TABLE I

AMINO ACID SEQUENCE AND CHARGE OF SYNTHETIC PEPTIDES AT NEUTRAL pH

Peptide	*Amino acid sequence*	*Charge*
Anti-LY	Glu–Ile–Phe–Pro–Gly–Asn–Ser–Lys–Thr–Tyr–Tyr–Ala–Glu	−1
P7	Ala–Val–Leu–Glu–Arg–Ala–Ala–Arg–Ser–Val–Leu–Leu–Asn–Ala–Pro[a]	+2
P26	Ser–Thr–Leu–Leu–Pro–Pro–Glu–Leu–Ser–Glu–Thr–Pro–Asn–Ala–Thr[a]	−1
P36	Glu–Leu–Ala–Pro–Glu–Asp–Pro–Glu–Asp–Ser–Ala–Leu–Leu–Glu–Asp[a]	−6

[a] C-terminal amide group.

(Pharmacia, Uppsala, Sweden) converted into the free thiol form by removing the 2-thiopyridyl protecting groups by treatment with dithiothreitol according to the instructions of the manufacturer. Four equal portions of the NPys-cysteinyl peptide were added to a column with 50 mg of thiopropyl-Sepharose containing 3.75 μmol of SH groups. The liberation of the yellow reaction product, 3-nitro-2-thiopyridone, was monitored after each addition. The eluate was colourless after the fourth addition, indicating that an excess of peptide had been used and that coupling of 6.5 mg of Cys(NPys) anti-LY peptide had been achieved. The column material was mixed with Sepharose 4B to give a column volume of 1.6 ml.

Three other peptides (analogous to the amino acid sequence of herpes simplex glycoprotein D) were synthesized as peptide amides (see Table I) according to the method of Houghten[6] and were used to prepare control affinity adsorbents. Peptides (2 mg) were coupled to 0.4 g of Tresyl-activated Sepharose 4B (Pharmacia, Uppsala) in 0.1 *M* $NaHCO_3$–Na_2CO_3 (pH 8.5) containing 1 *M* sodium chloride according to the instructions of the manufacturer. The percentage coupling was determined by reversed-phase (RP) HPLC of the peptide solution before coupling and the eluate after coupling. Peptides P7, P26 and P36 were coupled for 35, 55 and 63%, respectively.

Chromatography

Affinity chromatography was carried out at room temperature at a flow-rate of 9 ml/h during application of samples and 18 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.

RP-HPLC was performed with a system from LKB (Bromma, Sweden) 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, Düren, F.R.G.). Proteins and peptides were eluted with a 20-min gradient from 10% acetonitrile in 0.1% TFA to 60% acetonitrile in 0.09% TFA. The absorbance was monitored at 214 nm.

Proteins

Hen egg-white lysozyme and bovine ribonuclease A were obtained from Boehringer (Mannheim, F.R.G), bovine serum albumin, ovalbumin and carbonic anhydrase from Sigma (St. Louis, MO, U.S.A.) and foetal calf serum from Gibco (Paisley, U.K.).

RESULTS AND DISCUSSION

Lysozyme is a relatively basic protein with an isoelectric point of 11. It may show interaction with hydrophilic groups present in Sepharose. Initial experiments showed that such interactions could be abolished by using 0.05 *M* sodium thiocyanate in the

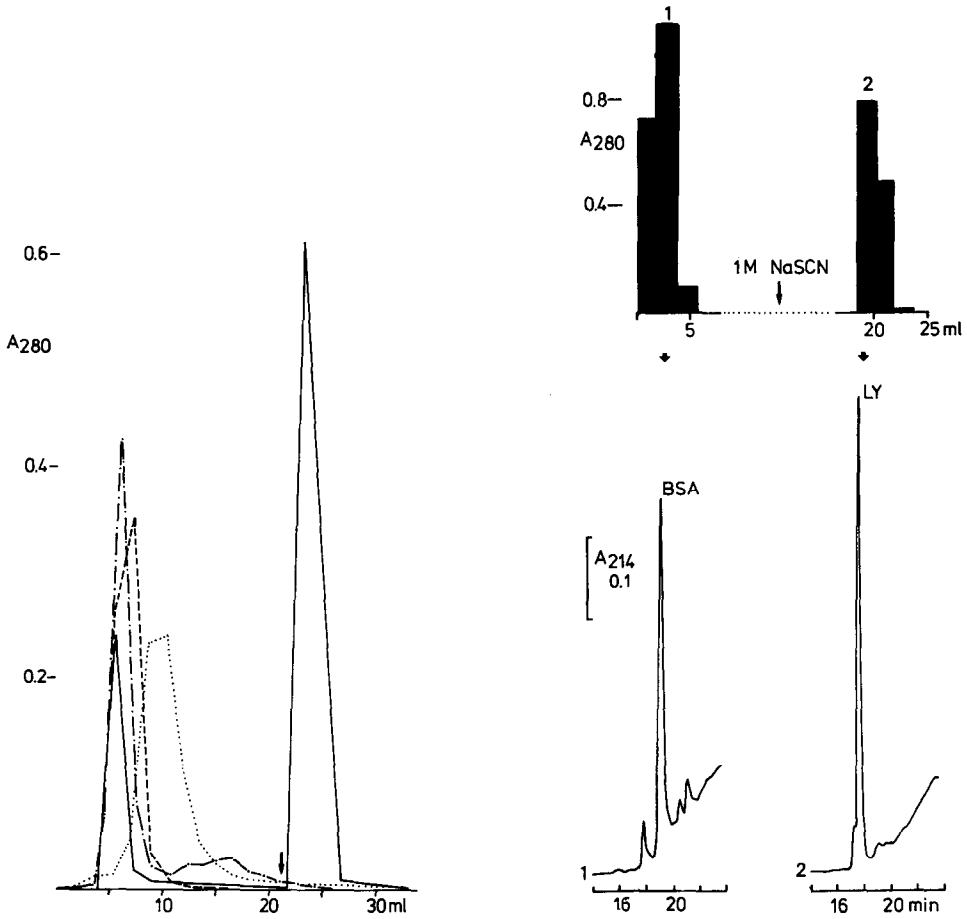

Fig. 2. Affinity chromatography of lysozyme on adsorbents with different peptide ligands. Synthetic peptides(Table I) were coupled either to thiopropyl-Sepharose (the anti-LY peptide) or to Tresyl-activated Sepharose (three herpes simplex virus glycoprotein D peptides). Amounts of 0.8 and 1.6 mg of lysozyme were applied to the herpes virus peptide adsorbents and the anti-LY adsorbent, respectively. 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 the elution with 1 *M* sodium thiocyanate in the same buffer. The flow-rate was 9 ml/h during application of the sample and 18 ml/h during chromatography. The absorbance was measured at 280nm. ——— = Anti-LY; — . — . — = P7; – – – = P26; ····· = P36. The charge and the amino acid sequence of the peptides are given in Table I.

Fig. 3. Affinity chromatography with the anti-LY adsorbent. A 1.6-mg amount of lysozyme in 10-fold diluted foetal calf serum was applied to the column. Elution conditions as in Fig. 2. Volumes of 100-μl of the indicated fractions 1 and 2 were analysed by RP-HPLC (bottom). BSA = Bovine serum albumin; LY = lysozyme.

starting buffer. Under these conditions, lysozyme did not bind to Sepharose, deprotected thiopropyl-Sepharose or deactivated Tresyl-Sepharose. Fig. 2 shows the elution pattern obtained after application of 1.6 mg of lysozyme to the anti-LY column and 0.8 mg of lysozyme to three control columns. Lysozyme was slightly retarded on the acidic column (P36; peptide charge before coupling, −6) and not at all on the other two control columns (P26 and P7; peptide charge, −1 and +2, respectively). Elution with 1 *M* sodium thiocyanate did not result in further peaks. In contrast, most of the lysozyme (77%) was specifically eluted by 1 *M* sodium thiocyanate from the anti-LY column (solid line, Fig. 2). Further experiments showed that lysozyme could be eluted between 0.15 and 0.25 *M* sodium thiocyanate from this column.

Fig. 3 shows the elution pattern when lysozyme in 10-fold diluted foetal calf serum was applied to the anti-LY column. Fractions were analysed by RP-HPLC (Fig. 3, bottom). The results show that the main component of the serum, bovine serum albumin, did not bind to the column and that lysozyme was specifically eluted by 1 *M* sodium thiocyanate.

In Fig. 4 the elution pattern is shown after affinity chromatography of a mixture of reference proteins including lysozyme. RP-HPLC analysis of fractions that were eluted with the starting buffer containing 0.05 *M* sodium thiocyanate showed that bovine serum albumin, carbonic anhydrase, ovalbumin and a small amount of

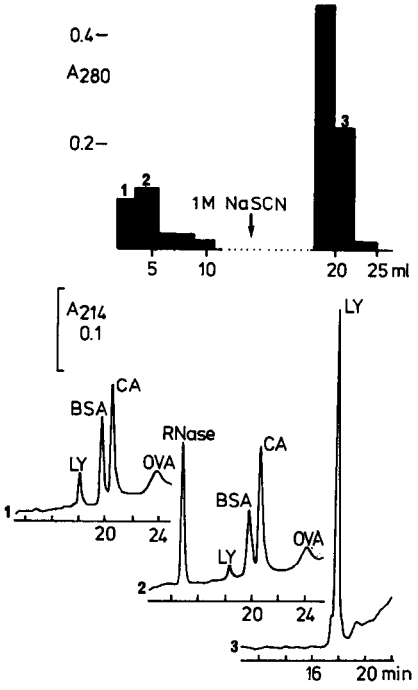

Fig. 4. Affinity chromatography with the anti-LY adsorbent. A mixture of 0.8 mg of lysozyme (LY) and 0.05 mg of pancreatic ribonuclease (RNase), bovine serum albumin (BSA), carbonic anhydrase (CA) and ovalbumin (OVA) was applied to the column. Elution conditions as in Fig. 2. Aliquots of 100 μl of the indicated fractions 1, 2 and 3 were analysed by RP-HPLC (bottom).

lysozyme did not bind to the column. Ribonuclease was slightly retarded and was eluted together with other proteins in fraction 2. Most of the lysozyme (95%) was eluted with 1 *M* sodium thiocyanate (*e.g.*, fraction 3).

The relatively small amounts of lysozyme which were eluted with 0.05 *M* sodium thiocyanate (Figs. 2–4) were not the result of overloading, as the capacity of the anti-LY column was at least 4.7 mg of lysozyme. Whether the presence of bound and unbound lysozyme can be explained by a pseudoequilibrium[7] between lysozyme and anti-LY remains to be investigated. Earlier studies[8,9] showed that after reduction of an immunoglobulin molecule by protein engineering to the variable region of the H and L chain, antigen binding properties could be retained. In the study by Huston *et al.*[8], a biosynthetic antibody incorporating the variable domains of an anti-digoxin monoclonal antibody in a single polypeptide chain (M_r = 26 354) did bind digoxin a factor of 6 less strongly than the intact monoclonal antibody. In the study by Bird *et al.*[9], similar single-chain constructs were made directed against bovine growth hormone and fluorescein. Recently, Ward *et al.*[10] showed that single variable regions, especially those from the H chain, could bind the antigen with good affinity. Williams and co-workers[11,12] showed that further reduction of the size of the variable domains was possible with retention of antigen-binding properties. They were studying the reovirus–reovirus receptor system. Antibodies were produced against reovirus and thereafter antibodies were made against the anti-reovirus antibodies. The amino acid sequence of the latter antibody was determined and peptides were synthesized with the amino acid sequence of the hypervariable segments. One of these peptides showed reovirus receptor-binding properties.

In our study we were aided by the availability of a model of the complex between an anti-lysozyme monoclonal antibody (Gloop 2) and lysozyme[3]. The variable segment of Gloop 2 with the largest number of contact residues with lysozyme was selected for synthesis. To avoid coupling to the ε-NH_2 of lysine at position 8 of the anti-LY peptide, it was coupled after elongation with NPys-Cys with its N-terminus to thiopropyl-Sepharose using the 3-nitro-2-pyridinesulphenyl protection–activation method of Drijfhout *et al.*[5]. This allowed contact of the amino acid residues of the anti-LY peptide with lysozyme, resulting in a sufficient number of interactions, *i.e.*, in binding of lysozyme.

These results show that immunoaffinity chromatography with a synthetic peptide ligand which mimics part of the antigen-binding site is possible. In this particular instance the selection of the peptide was aided by the availability of a tertiary structure model of the protein antigen and the amino acid sequence of monoclonal antibodies directed against the antigen. In general, less information would be sufficient in order to design a peptide ligand for the purification of a particular protein. The amino acid sequence of the variable regions of a monoclonal antibody can be determined by sequencing of immunoglobulin mRNA[13] and, as the variable regions do contain a number of invariant residues, the hypervariable segments can be located easily. A set of overlapping synthetic peptides from these antigen-binding regions should then be tested for the ability to bind the protein antigen. Immunoaffinity adsorbents based on this principle may be a useful tool in the selective purification of a protein.

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