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Application of reversible biotinylated label for directed immobilization of synthetic peptides and proteins: isolation of ligates from crude cell lysates

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Abstract: Chaperonin 10 protein from *Rattus norvegicus* (Rat cpn10) has been reported to bind chaperonin 60 from *Escherichia coli* (GroEL) in an ATP-dependent manner. Chemically synthesized Rat cpn10 was immobilized in a defined orientation to agarose-bound monomeric avidin using a reversible biotinylated affinity label (**1**), attached to the N^{α}-terminal residue. The resulting affinity chromatographic matrix was then used to isolate binding proteins from a crude cell lysate. Following affinity separation the bound ligand and ligate was released by treatment with organic base. Rat cpn10 was prepared using a highly effective synthetic protocol involving HBTU/HOBt activation and capping with *N*-(2-chlorobenzyloxycarbonyloxy) succinimide to terminate unreacted amino groups. The biotinylated Fmoc-based molecule (**1**) was introduced specifically onto the N^{α}-terminal amino acid as the succinimidyl carbonate, before final cleavage and deprotection of side-chain protecting groups using a low-TFMSA/high-HF procedure. Crude biotinylated Rat cpn10 (Rat cpn10 + **1**) was immobilized on monomeric avidin with a binding efficiency of approximately 75% and unlabelled truncated/capped impurities eluted off the column with buffer. The biotinylated Rat cpn10–avidin affinity matrix was then used to isolate GroEL from a crude cell lysate. The identity of the purified protein was confirmed by SDS–PAGE and binding to a specific anti-GroEL monoclonal antibody (MoAb). These results extend the applicability of the biotinylated label (**1**), providing a reversible non-covalent anchor for immobilization of peptide and protein ligands, thus simplifying isolation of ligates and enabling recovery of synthetic material under mild conditions. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

Immobilization of peptide or protein ligands onto a solid support for affinity chromatographic separation has been described, using both covalent and non-covalent coupling chemistries. Covalent immobilization offers the advantage that very stable linkages can be formed that are unaffected by high salt concentrations and extreme pH [1]. Several covalent linkages have been described for the immobilization of heparin, which serve to illustrate the principal difficulties associated with affinity separations on a solid support. Previously, heparin had been immobilized through its uronic acid [2] or hydroxy groups [3] onto amino functionalized or cyanogen-activated supports, respectively. Since multiple positions within the heparin chain are bound to the support, coupling occurs non-directionally. As a result the binding between the ligand and its ligate can often be impaired due to steric

Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; HBTU, 2(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; PBS, phosphate-buffered saline; TFMSA, trifluoromethane sulphonic acid.

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hindrance around the binding sites [4]. Ideally, to obtain the most effective affinity chromatography matrix the ligand should be immobilized using only one point of contact with the solid support. In the case of heparin, it was found that specific directional immobilization through either the carboxyl or amino-terminal residue resulted in an eight-fold improvement in binding capacity of protamine [5].

Stepwise solid-phase peptide synthesis (SPPS) is ideally suited to the labelling of specific residues which can then be utilized for immobilization purposes. The residue could be an integral part of the sequence residue or attached to either the C- or N-terminal cysteine depending on whether or not binding capacity was compromised. The classical amino acid of choice is cysteine which is attached to a cysteine-containing resin via a disulphide bond [6], but is limited if other cysteine residues are present in the sequence. Ideally, a non-covalent interaction is preferable over a covalent one in order to facilitate recovery of valuable synthetic or recombinant peptides and proteins after affinity procedures. Metal ion affinity chromatography has been described for the immobilization of synthetic peptides, with binding occurring via the α-amino group [7]. The effectiveness of the methodology is reduced, however, if the sequence contains strong metal-binding amino acids (i.e. His, Trp or Cys). In addition the interaction is only effective over a narrow pH range.

Much research has been directed towards the exploitation of one of the strongest non-covalent interactions for affinity chromatography, i.e. that between avidin and biotin ($K_a = 10^{15}$) [8]. Furthermore, the biotin–avidin interaction is independent of amino acid composition and can be broken under mild conditions when using the monomeric form of avidin. Chemically synthesized peptides and proteins have been N^{α}-terminally labelled with biotin to aid purification of the final product [9–11]. However, the addition of biotin through an amide bond resulted in an irreversible modification of the peptide sequence [9] or relied on autocatalytic cleavage of the biotin-linker segment in order to obtain the correct sequence [10]. A reversible biotinylated label has been reported, but its application was described for the purification of relatively short peptide sequences [11].

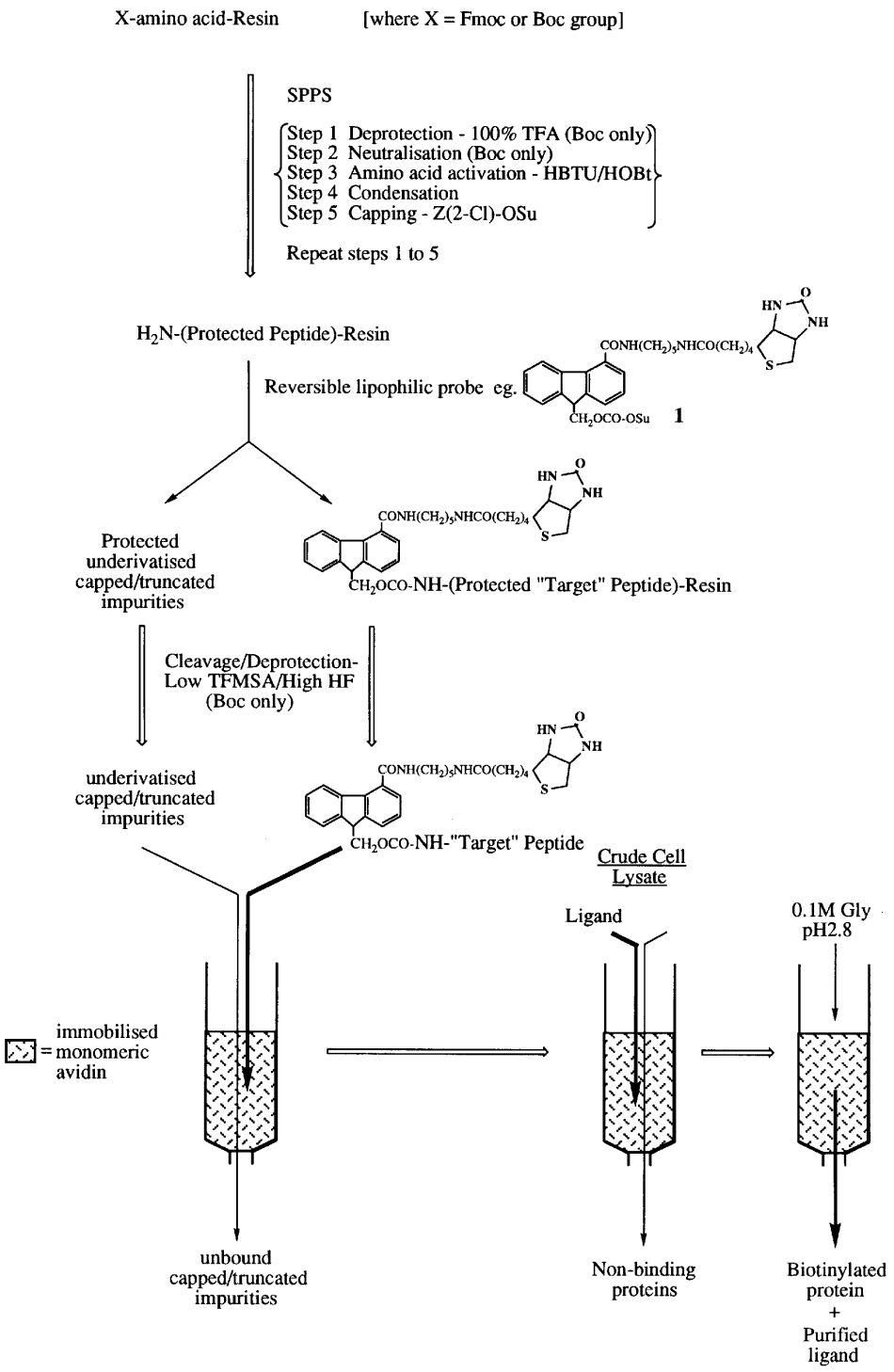
We have developed an integrated synthetic and purification procedure based on (i) highly efficient acylating reagents to optimize coupling yields, (ii) an effective capping procedure to eliminate deletion sequences and (iii) the incorporation of an affinity label, which has been labelled with reversible groups possessing specific chromatographic properties (e.g. hydrophobic or biotin), to the N^{α}-terminus of the target peptide [12–14]. Thus, peptide chains that have been labelled with a chromatographic label are easily separated from unlabelled impurities on appropriate media (e.g. RP or immobilized avidin). Quantitative removal of the affinity label is achieved using dilute aqueous base, which after desalting on RP media, yields the pure free peptide. In our previous work we have shown that the biotinylated label (**1**) was successfully used to purify the 46 residue β2-subunit from CD18 and the 101 residue mitochondrial chaperonin 10 protein from *Rattus norvegicus* (Rat cpn10), synthesized using both Fmoc [13] and Boc [14] chemistries, respectively.

We report here the use of the biotinylated label (**1**), when attached to a synthetic protein ligand, as a 'tool' for isolating ligates from crude cell lysates. Scheme 1 illustrates the general principles of stepwise SPPS, i.e. incorporation of **1** onto the N^{α}-terminus of the peptidyl-resin and subsequent immobilization of reversibly biotinylated protein for either purification purposes or isolation of binding proteins from cell lysates. To demonstrate this latter application N^{α}-terminally biotinylated Rat cpn10, which is known to bind and generate an active complex with prokaryotic chaperonin 60 from *Escherichia coli* (GroEL) [15–17], was used to isolate GroEL from a crude cell lysate.

MATERIAL AND METHODS

Analytical Methods

High-performance liquid chromatography (HPLC) was performed with a Model LC10 system (Shimadzu, Japan) equipped with a C_{18} reversed-phase (RP) column (150 × 4.6 mm I.D.) (Vydac, USA) using the following two solvent systems: 0.045% trifluoroacetic acid (TFA) in water (solvent A) and 0.036% TFA in acetonitrile (solvent B). Electrospray ionization (ESI) mass spectrometry was carried out on a Model 700 spectrometer (Finnigan MAT, USA). SDS–PAGE and protein blotting were performed on PhastSystem and PhastTransfer systems (Pharmacia, Sweden), respectively. Alkaline phosphatase (Protoblot, USA) test was performed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (Promega, USA). Recombinant GroEL was obtained from Boehringer Mannheim (Penzberg, Germany).



Scheme 1 General scheme illustrating the application of reversibly biotinylated protein ligands, synthesized using optimized chemical protocols, for the isolation of ligates from crude cell lysates.

Peptide Synthesis, Introduction of Biotinylated Probe (1) and Cleavage

Full details of the chemical synthesis of Rat cpn10 using HBTU/HOBt coupling cycles with capping and the procedures for introducing the biotinylated molecule are reported elsewhere [14, 16]. In brief, single HBTU/HOBt-mediated couplings were used throughout, except for Asn, Gln and Arg residues which were double coupled. A capping procedure using *N*-(-2-chlorobenzyloxycarbonyloxy) succinimide was performed after the addition of each amino acid to terminate any deletion sequences [18]. The protein was synthesized on PAM resin pre-derivatized with the first amino acid. At the end of the synthesis, which was completed in 2.5 days, the N^{α}-terminal protecting group was removed and the biotinylated probe introduced as the succinimidyl carbonate (**1**), using a four-fold excess of **1**. Coupling was complete after 90 min as determined by quantitative ninhydrin analysis [19]. Some 50 mg of Rat cpn10 + **1** and 50 mg of unlabelled peptidyl-resins were cleaved using a low-TFMSA/high-HF protocol [20] in the presence of scavengers *p*-cresol, dimethyl sulphide, TFA, TFMSA (4 : 15 : 25 : 5) at 0°C for 2 h and *p*-cresol, *p*-thiocresol, HF (4 : 1 : 50) at 0°C for 1 h, respectively. HF was removed under vacuum and the cleaved proteins precipitated with dry diethyl ether. The solid was filtered and redissolved in 20% acetic acid solution before lyophilization which yielded 28.3 mg (82%) of the peptidyl-resin treated with **1** and 25.2 mg (85%) crude untreated Rat cpn10 resin.

Affinity Chromatography (General Method)

Preparation of Cell Lysate. *Escherichia coli* cells (pGT 3270) were suspended in 2 ml of PBS buffer pH 7.2, containing $CaCl_2$ (0.1 g/l). The suspension was sonicated at 0°C for 10 s, allowed to cool for 30 s and the procedure repeated for 10 cycles. After sonication the suspension was centrifuged (15,000 × g for 5 min) to remove cell debris and the supernatant transferred to the ultrafiltration cell. Under positive pressure (10 p.s.i.) the supernatant was reduced in volume using a membrane (cutoff 100,000). In order to eliminate unwanted low molecular weight proteins and endogenous ATP, additional buffer was placed in the cell and the ultrafiltration continued until a final volume of approximately 3 ml was obtained. The remaining supernatant was removed after repeating the dilution procedure several times.

Affinity Chromatography. An empty glass column (50 × 5 mm I.D.) was loaded with 3 ml monomeric avidin immobilized on beaded agarose (Pierce, USA). Non-reversible biotin binding sites were blocked by passing 2 mM *d*-biotin in PBS through the column, using a peristaltic pump at a flow rate of approximately 1 ml/min. Reversibly bound biotin was then removed from the desired low-affinity binding sites with 0.1 M glycine, pH 2.8. The column was re-equilibrated with PBS pH 7.2 buffer containing $CaCl_2$ (0.1 g/l), 7 mM $MgCl_2$ and 2 mM ATP. Some 10 mg of crude Rat cpn10 + **1** was dissolved in 200 µl 8 M guanidine hydrochloride and then diluted to 2 ml with PBS pH 7.2 buffer containing $CaCl_2$ (0.1 g/l) and 2 mM ATP. Any undissolved material was removed by filtration before loading onto the avidin column. Unbound protein was eliminated by pumping buffer through the column. The supernatant from the ultrafiltration cell was diluted with 2 ml of PBS pH 7.2 buffer containing $CaCl_2$ (0.1 g/l), 7 mM $MgCl_2$ and 2 mM ATP, and loaded onto the equilibrated Rat cpn10 + **1**–avidin column. After removing non-binding material with buffer, Rat cpn10 together with any bound protein were released by treating the resin with 5% aqueous triethylamine. The basic solution was neutralized with 10% acetic acid before desalting on RP media.

Characterization

SDS–PAGE. SDS–PAGE analysis was performed on PhastSystem (Pharmacia, Sweden) Homogenous 20 gels and visualized using silver stain.

Anti-GroEL Monoclonal Antibody Binding. Proteins were separated on a PhastSystem Homogenous 20 gel using recombinant GroEL as standard and then blotted onto a PVDF membrane (Millipore, Japan) soaked in 25 mM Tris, 192 mM glycine, 20% methanol at pH 8.3, using the PhastTransfer instrument (Pharmacia, Sweden). The membrane was then incubated for 60 min with PBS (1% BSA and 0.05% Tween 20, pH 7.2). A stock solution of 200 µg of anti-GroEL monoclonal antibody (9A1) (StressGen, Canada) in 200 µl of PBS was prepared. For biological studies a 1/500 dilution of stock solution using PBS (1% BSA and 0.05% Tween 20, pH 7.2) was made and the membrane incubated for a further 60 min. The membrane was then washed thoroughly with PBS (0.05% Tween 20, pH 7.2). To visualize any proteins that had bound the anti-GroEL MoAb, a two-step procedure was performed. In the first step the membrane was incubated with

alkaline phosphatase-goat anti-mouse IgG (Zymed, USA). After washing the membrane thoroughly with PBS buffer (0.05% Tween 20, pH 7.2), it was incubated in 5 ml 100 mM Tris–HCl buffer containing 100 mM NaCl, 5 mM $MgCl_2$ at pH 9.5, together with 33 μl nitroblue tetrazolium and 16.5 μl 5-bromo-4-chloro-3-indoyl phosphate, for 60 min. The appearance of purple bands was positive for anti-GroEL MoAb binding.

RESULTS AND DISCUSSION

Classical peptide and protein biotinylation procedures involve the derivatization of any exposed free amino groups, usually those of lysine residues, thus resulting in non-directional binding to the avidin column. The methodology described here offers distinct advantages in this respect since the introduction of biotin is controlled and is performed specifically on the N^{α}-amino group of the peptide chain at the end of chemical synthesis and before final cleavage from the resin. Instead of direct covalent attachment of biotin we have designed a chromatographic affinity label (**1**) that possesses (i) a base-labile fluorenylmethoxy/carbonyl (Fmoc) group so that unlabelled protein can be recovered if desired and (ii) a spacer group consisting of five alkyl chains to reduce the possibility of steric hindrance from either the Fmoc moiety or protein that could reduce binding efficiency [5].

Peptide Synthesis

We previously reported the synthesis of Rat cpn10 and the addition of a biotinylated molecule (**1**) to the N^{α}-terminus of the peptidyl-resin [14]. Briefly, single HBTU/HOBt-mediated coupling was used except for Asn, Gln and Arg residues which were double coupled. Unreacted amino groups were terminated using a highly efficient capping procedure, at the end of each cycle, requiring the use of *N*-(-2-chlorobenzyloxycarbonyloxy) succinimide [18]. The 101 residue protein was completed in 2.5 days at which time the N^{α}-terminal protecting group was removed and the biotinylated label (**1**) introduced as the succinimidyl carbonate. Some 50 mg of Rat cpn10 + **1** and 50 mg of unlabelled peptidyl-resins were cleaved using a low-TFMSA/high-HF protocol [19]. An aliquot of each of the two crude cleavage products was analyzed by RP-HPLC and gave the chromatograms shown in Figure 1.

Directed Immobilization of Biotinylated Rat cpn10 + 1

10 mg of crude Rat cpn10 + **1** was dissolved in 8 M guanidine hydrochloride (Gdn.HCl). The solution was then diluted to 1 M Gdn.HCl was PBS pH 7.2 buffer, containing Mg^{2+}/Ca^{2+} and ATP. The presence of a heptameric structure was confirmed by gel filtration chromatography [17]. Rat cpn10 + **1** was passed through the pre-equilibrated immobilized monomeric avidin column at a low flow-rate and unbound material was eluted off the column with PBS pH 7.2 buffer, containing Mg^{2+}/Ca^{2+} and ATP. An aliquot of the latter was analyzed by RP-HPLC and the resulting chromatogram (Figure 1C) showed that peak 2 in Figure 1B, corresponding to Rat cpn10 + **1** had been retained by the avidin column [3]. From an examination of peak heights it would appear that the binding efficiency was approximately 75%. Improved binding could possibly be achieved by incubating crude protein mixture with the immobilized avidin matrix over an extended period or using a longer spacer group between biotin and the protein.

It was previously reported that GroEL formed a functional Mg–ATP dependent complex with Rat cpn10 from recombinant sources [15]. This observation was confirmed using synthetic Rat cpn10 that was not N^{α}-terminally acetylated [17]. Furthermore, when an enolase refolding experiment was performed using synthetic Rat cpn10 and GroEL, 86% recovery of enzyme activity was obtained after 6 min, compared with 92% achieved with chaperonin 10 from *E. coli* (GroES) [15].

Affinity Chromatography

A preliminary experiment was required to confirm that Rat cpn10 + **1** protein was capable of binding to GroEL in the presence of ATP, when immobilized on the avidin column. Thus, 20 μl of recombinant GroEL (10 mg/ml) was diluted to 500 μl with PBS buffer pH 7.2, containing Mg^{2+}/Ca^{2+}, ATP and loaded onto an avidin column which had been treated with another 10 mg of Rat cpn10 + **1**, as described previously. Unbound material was washed off the column with buffer and then the column was treated with dilute organic base to release bound protein. SDS–PAGE confirmed that GroEL had complexed with Rat cpn10 + **1**, as demonstrated by the presence of a band (lane 4, Figure 2A) possessing the same migration characteristics as a reference sample of recombinant GroEL (lane 2, Figure 2A). The same experiment was

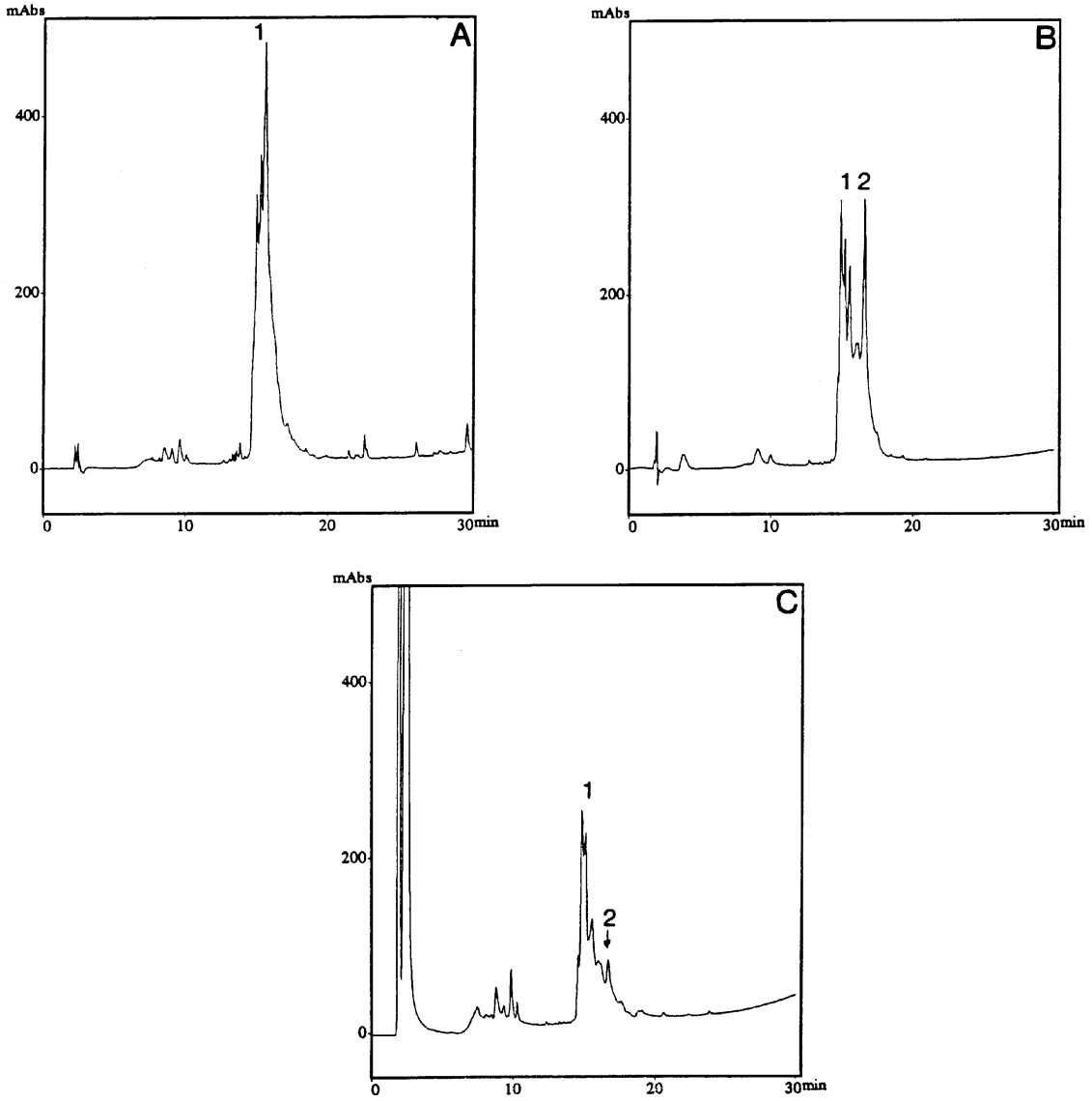


Figure 1 Analytical RP-HPLC of Rat cpn10 on C_{18} with 0 to 100% in 30 min acetonitrile gradient, flow 1 ml/min and detection at 220 nm. Panel A represents crude unlabelled Rat cpn10. Panel B is crude Rat cpn10 labelled with **1**. Panel C is non-binding material after elution through immobilized monomeric avidin column. Peaks labelled 1 represent unlabelled impurities, while those labelled 2 correspond to Rat cpn10 + **1**.

repeated in the absence of ATP and the lack of a band at approximately 67 KDa (lane 3, Figure 2A) was consistent with previous data that complex formation between Rat cpn10 and GroEL is ATP dependent [4]. Similarly, the presence of a single band of 11.6 KDa also served to eliminate the recurrence of other non-specific interactions with the avidin/agarose column. These results showed that N^{α}-terminal immobilization of Rat cpn10 did not compromise those structural features required for recognition and binding of Rat cpn10 to GroEL.

Isolation of GroEL From Crude Cell Lysate

The potential of this technique was evaluated using immobilized Rat cpn + **1** and a cell lysate prepared from *E. coli*. The pGT 3270 *E. coli* cells were

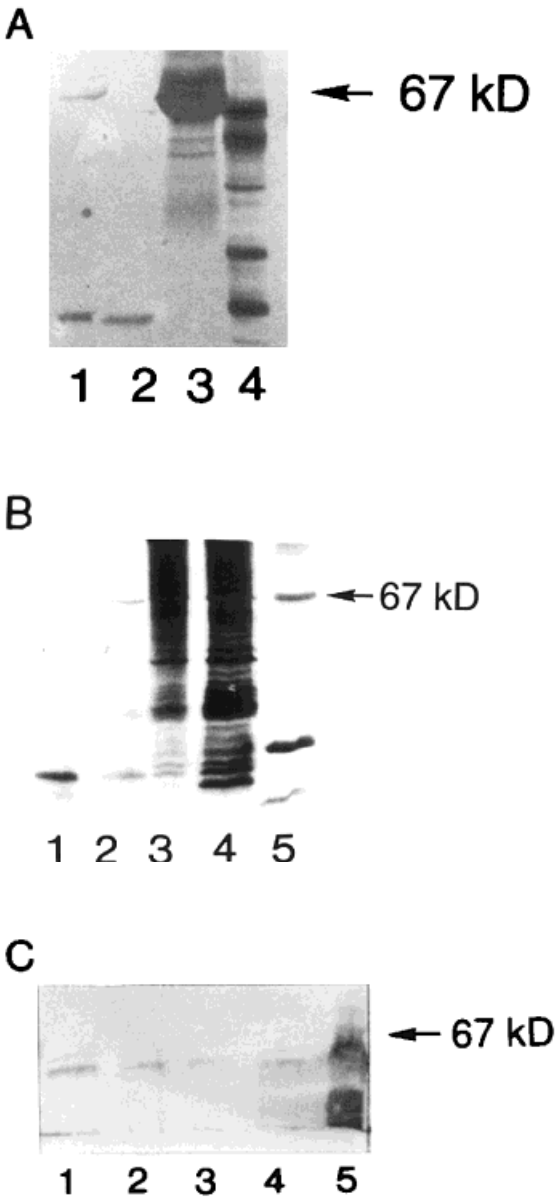


Figure 2 SDS–PAGE on Homogeneous 20 Phastgel
Panel (A) shows recombinant GroEL retained by Rat cpn10 + **1**–avidin column after elution with base (line 1), purified Rat cpn10 (lane 2), recombinant GroEL reference sample (lane 3) and low molecular weight markers (lane 4). Panel (B) represents purified Rat cpn10 (lane 1), protein eluting off monomeric avidin column after treatment with base (lane 2), crude *E. coli* cell lysate after (lane 3); before (lane 4) ultrafiltration and recombinant GroEL and GroES markers (lane 5). Panel (C) shows binding of anti-GroEL MoAb of 67 KDa band corresponding to GroEL with decreasing concentration of sample (lanes 1–4) after SDS–PAGE and Western blot procedures. Lane 5 corresponds to binding of anti-GroEL MoAb reference sample of recombinant GroEL.

suspended in PBS (Ca^{2+}) pH 7.2 buffer and sonicated to release cellular proteins. Unwanted cellular debris was removed by centrifugation and the supernatant separated. Since any GroEL could be present in a complexed form with endogenous GroES, it was first necessary to remove the latter by ultrafiltration. Thus, the supernatant containing the cellular proteins was placed in the ultrafiltration cell and filtration performed through a membrane (cutoff 100 000) under positive pressure. This procedure effectively removed smaller molecular weight proteins such as the heptameric GroES structure (~70 KDa), as well as the Mg–ATP required to maintain GroEL and GroES in their complexed form. A sample of *E. coli* lysate was run on SDS–PAGE both before (lane 2, Figure 2B) and after (lane 3, Figure 2B) ultrafiltration and showed that the procedure had resulted in a significant reduction in the concentration of lower molecular weight proteins, as expected. The remaining supernatant was then loaded onto the equilibrated immobilized avidin–Rat cpn10 + **1** column and unbound material eluted off the column with buffer. To release bound protein, as well as free Rat cpn10, the column was treated with base as described previously. SDS–PAGE analysis clearly showed a band of approximately 67 KDa corresponding to GroEL, together with the expected 10 KDa band due to the presence of Rat cpn10 + **1** (lane 4, Figure 2B).

It was then necessary to confirm that it was GroEL that had been isolated and not another protein with an apparent molecular weight similar to that of GroEL. Thus, another SDS–PAGE was run and the protein blotted onto a PVDF membrane. The proteins that had transferred onto the membrane were then incubated for 60 min with PBS containing BSA and Tween 20, followed by an additional 60 min with a diluted sample of 9A1 MoAb specific for GroEL in the same buffer. The membrane was then washed thoroughly with PBS containing Tween 20. To visualize any proteins that had bound the anti-GroEL MoAb, the membrane was incubated with alkaline phosphatase-goat anti-mouse IgG, followed by the phosphatase substrates. The lane containing the recombinant GroEL reference sample gave a positive reaction as indicated by a band at approximately 67 KDa (lane 5, Figure 2C). Similarly, the protein that had been isolated from the *E. coli* cell lysate and possessed an apparent molecular weight of 67 KDa also reacted with the anti-GroEL MoAb (lanes 1–4, Figure 2C), thus indicating that the protein was GroEL. The broad band observed below that of monomeric recombinant GroEL at 67 KDa

represent aggregated forms of GroEL and were also found to cross-react with the MoAb.

CONCLUSIONS

We have utilized the specific Mg–ATP dependent complex formation between Rat cpn10 and GroEL as a model system to demonstrate the application of a directed immobilization procedure that enables the recovery of synthetic material using mild conditions. Rat cpn10 was chemically prepared and labelled selectively on the N^{α}-terminal residue with a reversible biotinylated molecule (**1**). The biotinylated Rat cpn10 + **1** was immobilized on a beaded agarose–avidin matrix from a crude cleavage mixture with approximately 75% efficiency. The data served to show that even in its biologically heptameric structure the biotin group, when attached to Rat cpn10, was available for binding with the avidin support. Furthermore, the directed orientation of the heptameric complex on the avidin matrix did not compromize those residues required for complex formation with GroEL. Confirmation that GroEL had been isolated from a crude cell lysate was shown by a band that co-migrated with a reference sample of GroEL on SDS–PAGE. The band with apparent molecular weight of 67 KDa was also recognized by anti-GroEL MoAb.

This study suggests an additional use of the reversible biotinylated molecule (**1**) as an alternative means of directed immobilization of synthetic peptide and protein ligands to allow the screening of crude lysates for isolation of unknown binding ligates.

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