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Microscale characterization of the structure–activity relationship of a heparin-binding glycopeptide using affinity capillary electrophoresis and immobilized enzymes

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Abstract

A heparin-binding glycopeptide (T3) from human serum amyloid P component was characterized by taking advantage of two important features of capillary electrophoresis: the low sample consumption and the possibility of doing on-line binding studies. Incubations with neuraminidase and proteolytic enzymes were carried out with enzymes immobilized on paramagnetic microbeads. Affinity capillary electrophoresis subsequently was used to characterize T3 and its fragments with respect to heparin binding. We find that an intact glycan moiety makes the C-terminal part of T3 relatively resistant to chymotryptic clevage. This protection is lost upon desialylation. Also, the C-terminus of T3 is involved in heparin binding while the N-terminal part of the molecule has no appreciable binding activity. The micromethods presented here make it feasible to perform structure–function studies even on the small amounts of analytes that are typically available when working with glycopeptides from natural sources.

Keywords: Structure–activity relationships; Affinity capillary electrophoresis; Immobilized enzymes; Glycopeptides; Peptides; Heparin

1. Introduction

In contrast to unmodified peptides glycopeptides are currently not readily synthesized. Biomolecular studies of glycopeptides therefore often rely on the use of fragments isolated from purified parent glycoproteins. As a consequence their availability typically is limited and analytical methods that consume as little material as possible are therefore needed. Capillary electrophoresis (CE) is one such micromethod that is well suited for the analysis of scarce quantities of material (pico- to femtomole amounts typically required for each analysis) [1,2]. In addition to its minimal sample consumption CE is also notable for the very high separation efficiencies that can be obtained even under non-denaturing conditions. This means that quantitative and qualitative binding studies can be performed during the separation. This approach has been called affinity CE (ACE) [3–8].

We have previously used ACE to identify and characterize peptides from the structure of the serum protein amyloid P component that interact with heparin [9]. Serum amyloid P component (AP) is a DNA-, heparin-, and lipopolysaccharide (LPS)-binding protein of unknown physiological function that also appears to bind to all types of amyloid deposits [10]. We identified a heparin-binding tryptic AP component (T3) that is a cysteine-containing glycopeptide of 25 amino acid residues containing one N-linked biantennary complex type oligosaccharide

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chain [11]. The heparin binding of both the natural and a synthetic non-glycosylated form of T3 has previously been characterized by ACE [8,9,12]. The function of the carbohydrate moiety of T3 is so far unknown but a contribution to the function of the parent protein may be inferred by its remarkably homogeneous structure as well as by its presence close to a ligand binding site in the protein [9,11].

Here, we extend the characterization of the natural glycopeptide, T3, isolated from the parent protein, by mapping the heparin binding activity to a subfragment of T3. We also assess the possible role of the carbohydrate moiety for heparin binding and for peptide stability using a combination of CE, ACE, and treatment with exoglycosidases and proteolytic enzymes immobilized on paramagnetic microbeads. The approach is a general method that makes it possible to perform detailed characterization of structure–function relationships in naturally derived peptide fragments available in scarce amounts.

2. Experimental

2.1. Materials

Human serum amyloid P component (AP) and the tryptic fragment of AP corresponding to amino acids 14–38 (T3) were isolated as described previously [9,13] except that in some cases the more stable [14] 4,4′-dithiodipyridine (Aldrich, Steinheim, Germany) was used instead of 2,2′-dithiodipyridine for the protection of the cysteinyl residue. The identity of the *S*-pyridylated T3 was verified by matrix-assisted laser desorption ionization (MALDI) MS using a PerSeptive Voyager Elite instrument by courtesy of P. Roepstorff, University of Odense, Denmark. Masses corresponding to intact T3 and T3 with the loss of one or two sialic acid residues were determined. Peptides were dried down by vacuum evaporation and reconstituted in water before use. Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas, sequencing grade was from Boehringer Mannheim Biochemica (Mannheim, Germany). Neuraminidase (E.C. 3.2.1.18) from *Clostridium perfringens* was from Oxford GlycoSciences (Abingdon, UK) and proteinase K (E.C. 3.4.21.14) was from Sigma (St. Louis, MO, USA). The marker peptide acetyl-PSKD-OH was synthesized by Schafer-N (Copenhagen, Denmark). Low-molecular mass (LMW) heparin, sodium salt (molecular mass approximately 5000) from porcine intestinal mucosa was from Calbiochem (La Jolla, CA, USA). Dynabeads M-280 tosylactivated were obtained from Dynal (Oslo, Norway). All other chemicals were analytical grade from Sigma.

2.2. Capillary electrophoresis

Electrophoresis buffers were 0.1 *M* sodium phosphate (mixtures of 0.2 *M* Na_2HPO_4 and 0.2 *M* NaH_2PO_4, diluted 1:1 with water) of the stated pH values. All buffer solutions were made up in HPLC-grade water (Merck, Darmstadt, Germany) and passed through 0.22 μm pore size filters (Corning, Corning, NY, USA) before use. CE was performed on a Beckman P/ACE 2050 using UV detection at 200 nm and a 50 μm diameter uncoated fused-silica capillary with 30 cm to the detector window (total length: 37 cm). Separations were carried out at 5 or 10 kV and the capillary cooling fluid was thermostatted at 25°C unless noted otherwise. The capillary was rinsed after electrophoresis for 1 min with each of the following: 0.1 *M* NaOH, water, and electrophoresis buffer. In affinity CE experiments a 1 min prerinse with electrophoresis buffer containing heparin was performed using a reverse flow direction (i.e., from the outlet to the inlet with the inlet end of the capillary in a vial containing water to minimize the risk of carry-over of heparin into the sample vial). The subsequent electrophoresis was done after immersing the capillary ends into a set of separate vials containing plain electrophoresis buffer. Very small volumes (down to 10 μl) of prerinse ligand-containing solutions were sufficient for this procedure.

2.3. Conjugation of enzymes to paramagnetic beads

Covalent coupling of enzymes to tosyl-activated Dynabeads was performed according to the instructions of the manufacturer. Briefly, 250 μl resuspended bead solution corresponding to $1.6 \cdot 10^8$ beads was washed twice for 1 min with 1 ml 0.1 *M* borate–NaOH, pH 9.5. The supernatant was removed after

exposure to a magnet for 1 min and the beads were then thoroughly resuspended in 250 μl of enzyme diluted in the borate buffer. Neuraminidase was added in an amount corresponding to 1 Unit and 0.1 mg chymotrypsin was added to another batch. After vigorous vortexing for 1 min the beads were incubated using a slow tilt rotation overnight at 4°C. After this the beads were washed as before and blocked with 2 *M* Tris–HCl, pH 9.5 for 2 h at room temperature by vortexing and slow tilt rotation as before. The beads were ready for use after washing in borate buffer.

2.4. Enzymatic degradation of T3 peptide

The purified and dried T3 glycopeptide isolated from approximately 0.25 mg trypsinized AP was resuspended in 50 μl water and a volume (between 2 and 10 μl) of the settled enzyme-conjugated Dynabeads was added to 5 μl of the T3 solution. In some experiments a 5 μl volume of suitable buffer (10 m*M* acetate, pH 5.5 for the neuraminidase and 10 m*M* Tris-HCl, pH 7.4 150 m*M* NaCl, 10 m*M* $CaCl_2$ for the chymotrypsin) was also added as indicated. Alternatively, the Dynabead–enzyme suspension was washed in the appropriate buffer before being added to the T3 solution. No attempts were made to change buffers between incubations. The mixing took place in polypropylene microtubes that were vigorously vortexed and kept in motion by slow tilt rotation. After a suitable time, the mixture was exposed to a magnet for 1 min and aliquots of the supernatant were analyzed by capillary electrophoresis. Chosen components of the incubation mixtures were desalted and purified by reversed-phase HPLC and characterized by MALDI-MS as above.

3. Results

The binding of heparin by T3 has previously been characterized by CE and other methods [9,13]. T3 is a glycopeptide corresponding to amino acids 14–38 of serum AP (Fig. 1). It is generated by fragmentation of AP by trypsin and is *S*-pyridylated to protect the Cys(36) residue which in native AP forms a disulfide bridge with Cys(95).

To investigate if the heparin binding activity could be mapped to a specific part of T3 it was attempted to subfragment T3 by treatment with chymotrypsin. This would theoretically [15] yield three fragments due to cleavage C-terminally to the Phe-residues that are present in T3, as indicated in Fig. 1. Chymotrypsin was conjugated to tosyl-activated paramagnetic beads (Dynabeads) to ensure easy removal of the enzyme after incubation. The activity of the chymotrypsin-Dynabead conjugate was ascertained by testing for cleavage of a synthetic peptide by reversed-phase HPLC (not shown). However, even though the area of the T3 peak diminished compared with the control no new peaks formed and no change in appearance time was observed after an overnight incubation with immobilized chymotrypsin (Fig. 2). Also, the usual heparin binding activity that is characteristic of T3 was demonstrated by an ACE experiment to be preserved after chymotrypsin incu-

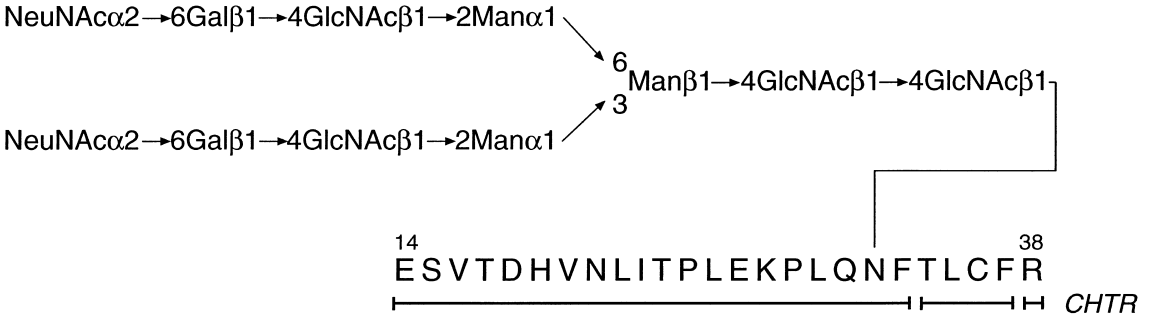

Fig. 1. Schematic structure of the T3 glycopeptide. It is purified as a fragment from a tryptic digest of denatured, reduced and *S*-pyridylated serum amyloid P component. One-letter amino acid residue abbreviations. Potential fragments generated by the action of chymotrypsin (CHTR) are indicated.

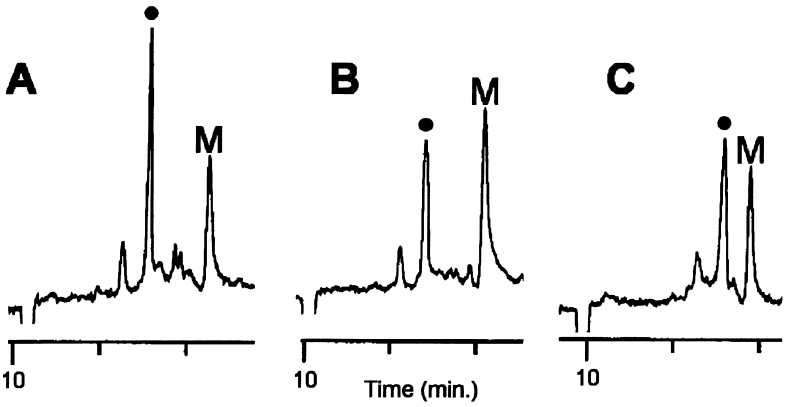

Fig. 2. T3 chymotrypsin resistance and LMW heparin reactivity in capillary electrophoresis. All separations carried out at 5 kV in 0.1 *M* phosphate, pH 8.1. In (A) a 4 s injection of purified T3 (marked with a (●)) is analyzed. M is the marker peptide at 0.01 mg ml^{-1} that was co-injected for 1 s. (B) is T3 after incubation overnight with chymotrypsin-Dynabeads, and (C) shows the increased appearance time of this T3 relative to the marker in the presence of 1 mg ml^{-1} LMW heparin added to the electrophoresis buffer. Detection at 200 nm.

bation (Fig. 2C). Under the conditions used T3 thus appeared to be resistant to cleavage by chymotrypsin despite the presence of susceptible peptide bonds. In a control experiment using the less specific protease proteinase K a number of fragments were readily generated from T3 (results not shown) but no attempts were made to characterize these further.

The effect of removing the sialic acids from the ends of the carbohydrate chains was next studied by CE and ACE (Fig. 3). CE clearly showed the distinctive effects of this enzyme on the glycopeptide charge with molecular forms of T3 compatible with 2 (i.e., intact), 1, and 0 sialic acids well separated (Fig. 3B). Almost complete desialylation as judged from the peak pattern and appearance time was achieved after an overnight incubation with neuraminidase-Dynabeads (Fig. 3C). The experiment in Fig. 3C was performed separately and under slightly different conditions than 3A and 3B and the migration of desialylated T3 (n-T3) is therefore faster than in 3B. The removal of sialic acids was verified by mass spectrometry (results not shown). In 3A it can be seen that a fraction (about 15% as judged by peak area measurements) of the starting material in this case had lost one sialic acid residue. It is not known if this is caused by the purification conditions that involve RP-HPLC under acidic conditions or if it may reflect the distribution of glycoforms of AP in vivo.

n-T3 had a preserved reactivity with LMW

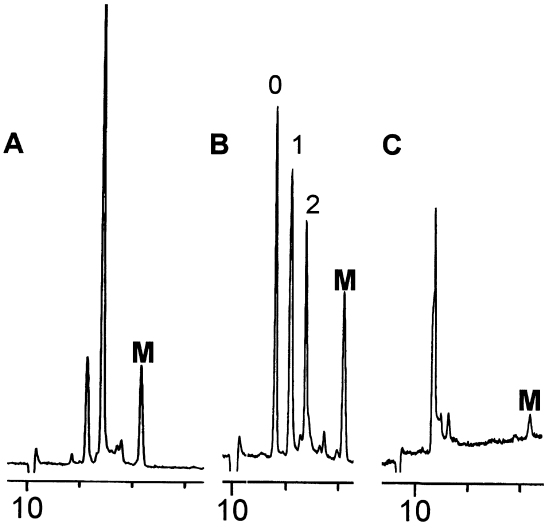

Fig. 3. Sialidase-Dynabead treatment of T3. Reaction monitored by CE. Neuraminidase beads in a volume of 2 μl were added to 20 μl T3+10 μl 10 m*M* acetate, pH 5.5. Sample aliquots (4 s injections) of the control incubation (A) and of the neuraminidase-treated T3 after overnight incubation at room temperature (B). The numbers 0, 1, and 2 indicate the numbers of sialic acids associated with the peptides of the individual peaks. (C) shows the result of a separate experiment in which a new batch of neuraminidase-conjugated beads was incubated with T3 overnight at room temperature using 10 μl neuraminidase beads to 5 μl T3+5 μl acetate, pH 5.5. A marker peptide (M) was co-injected for 2 s (1 s in (C)). Electrophoresis as given in Fig. 1 except that the electrophoresis buffer in (A) and (B) was 0.1 *M* phosphate, pH 7.85. Time scale in min.

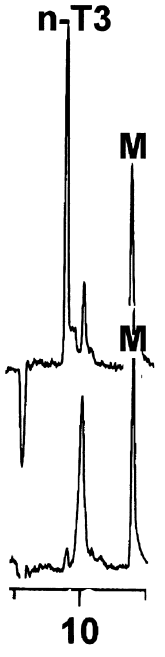

Fig. 4. Binding of desialylated T3 peptide to LMW heparin demonstrated by CE. After complete removal of sialic acids by incubation with neuraminidase-Dynabeads (cf. Fig. 3) the desialylated T3 (n-T3) was injected for 2 s and electrophoresed in the absence (upper panel) or the presence (lower panel) of 1 mg ml^{-1} LMW heparin added to the electrophoresis buffer. Conditions otherwise as given in Fig. 2 except that the capillary was not thermostatted in this experiment. Time scale in min.

heparin as illustrated by Fig. 4. The dissociation constant for the binding of n-T3 to heparin could not be determined directly using a series of preincubated samples as was done for intact T3 [13] because of the small amounts of material available. It did appear, however, that n-T3 shifted into complexed forms at lower concentrations of heparin than the fully sialylated peptide did, i.e., a 50% reduction of the area corresponding to the free n-T3 peak occurred at roughly half the concentration of LMW heparin that was necessary to exert the same effect on intact T3.

The susceptibility of the desialylated T3 (n-T3) to chymotrypsin degradation was next checked by CE after incubating n-T3 with chymotrypsin-conjugated beads (Fig. 5) under the same conditions that were used for chymotrypsin treatment of intact T3 (cf. Fig. 2). In contrast to the lack of change when intact T3 was treated with chymotrypsin the incubation of n-T3 with this enzyme yielded a new peak that migrated slower than n-T3 (Fig. 5B). This component had a molecular mass by MALDI-MS of 3917.98 which corresponds (expected value: 3917.39) to the mass of the large 20 amino acid fragment (amino acids 14–33) that is expected to form upon chymotrypsin treatment of T3 (cf. Fig. 1). The other expected fragments were not observed.

Thus, T3 becomes more susceptible to chymo-

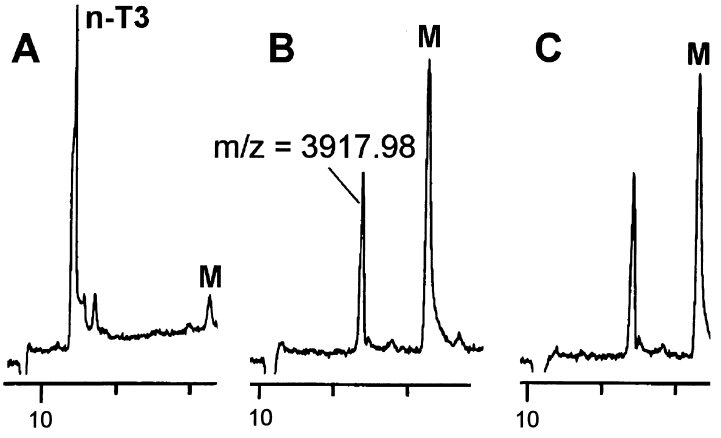

Fig. 5. Chymotrypsin treatment of desialylated T3 and LMW heparin reactivity of the main product. The desialylated T3 peptide in (A) (identical to Fig. 3C) was treated with chymotrypsin-beads overnight and then analyzed after 5 s injections together with the marker (M) in the absence (B) or the presence (C) of 1 mg ml^{-1} heparin added to the electrophoresis buffer. Mass spectrometry (MALDI-TOF) of the T3 fragment gave the indicated mass value. Conditions otherwise as given for Fig. 2. Time scale in min.

trypsin cleavage at the Phe(33)–Thr(34) bond after removal of the two sialic acids from the carbohydrate chain. The AA14-33 chymotryptic T3 fragment had no apparent binding activity towards LMW heparin because there was no migration shift when adding LMW heparin to the electrophoresis buffer in an ACE experiment (Fig. 5C). It was concluded that this fragment does not have a heparin-binding activity that is comparable to the activity of intact T3 and n-T3.

4. Discussion

The present work illustrates the feasibility of using capillary electrophoresis for affinity and structure studies of small molecules. The approach makes it possible to get results from scarce amounts of biological material. The use of immobilized enzymes makes it very easy to terminate reactions and to employ relatively high enzyme/substrate ratios without getting interference from material from the enzyme preparations in subsequent analyses. The drawbacks are that the amount of immobilized, active enzyme is unknown and that it is difficult to get a fully satisfactory mixing of beads and solution. Also, proper blocking of beads is very important or a peptide may get lost quickly through the formation of covalent bonds or by adsorption. The latter event may be counteracted by including moderate concentrations (0.1–0.2%) of non-ionic detergents, e.g. β-octylglucoside in the incubation mixtures.

The conclusions of the present study are summarized in Fig. 6. When an intact carbohydrate moiety is present on T3 it appears to offer protection against cleavage of the Phe(32)–Thr(33) bond. The protection may be due to the orientation of one of the arms [16] of the carbohydrate antenna along the chain as a result of electrostatic interactions between the positively charged Arg(38) and one of the two negatively charged sialic acid groups that terminate the carbohydrate chains. This would be even more pronounced in the intact protein where there is no C-terminus at Arg(38). We cannot exclude the possibility that other mechanisms account for the protective effects of the intact carbohydrate. It is not known if removal of just one sialic acid would be enough to render T3 susceptible to chymotrypsin but

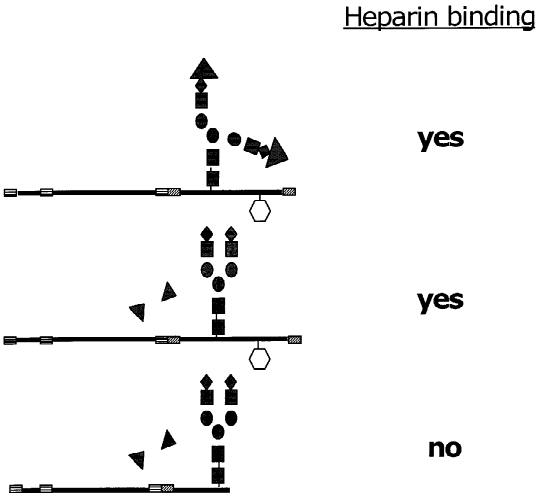

Fig. 6. Schematic figure of the conclusions of the present study. The structure of the glycan and the amino acid sequence are given in Fig. 1. Diagonally hatched boxes represent positively charged amino acid residues while horizontally hatched boxes are negatively charged residues. Upon release of the terminal sialic acid residues the T3 glycopeptide is still binding heparin but no longer protected against the action of chymotrypsin that generates the long amino acids 14–33 fragment that is devoid of binding activity.

the main fraction of the parent protein in vivo appears to contain two sialic acid residues [11]. If charge interactions are important for maintaining a carbohydrate-dependent protection against proteolysis the removal of the *C*-terminal arginine residue by treating the T3 glycopeptide with carboxypeptidase B should also render it susceptible to chymotrypsin cleavage but this has not yet been examined. The results do not elucidate if Lys(28) – which is not cleaved by trypsin because it is followed by a proline – plays a role in charge interactions with the sialic acids.

The present study does show, in accordance with solid-phase binding inhibition and cell attachment experiments using synthetic peptides [9,17], that the six amino acids in the C-terminal part of the peptide are necessary for the heparin binding activity of T3 because the large chymotryptic peptide that do not contain this sequence could not be demonstrated to have activity. The tetrameric TLCF fragment (see Fig. 1) was not detected in our CE experiments possibly because it is not recovered in solution due to its hydrophobic character. Our results also support

the lack of microheterogeneity and the biantennary, sialic acid terminated chain structure of the glycan [11] because we find only slight, possibly artifactually, heterogeneity of the intact T3 with respect to charge in the CE analyses (i.e., 5–15% of forms that appear to have lost one sialic acid in different batches of intact T3) and only get two well-defined additional charge forms upon neuraminidase-treatment. With regard to the propensities of intact and desialylated T3 to interact with heparin it was found that desialylation augmented the interaction as would be expected when removing negative charges from a peptide that interacts with a highly negatively charged molecule.

T3 is unusual for a heparin-binding peptide in having no clusters of basic amino acids and it is not known if the heparin binding of AP is actually mediated in vivo by this peptide or if the binding is a reflection of another physiological function. In any case, the apparent importance of the glycan in protecting the site and the invariable nature of the glycan seem to indicate that the site is important for the function of amyloid P component.

Other studies using synthetic peptides corresponding to amino acids 27–39 of AP and inhibition binding assays have shown that LPS is strongly bound by the C-terminal half of T3 in a sequence-specific manner [10]. In contrast to the case with heparin these studies fail to demonstrate a dependence of the LPS binding activity on the nature of the *S*-protecting group and even demonstrated an increased binding of the *S*-carboxamidomethylated peptide. In contrast, binding experiments in capillary electrophoresis have demonstrated the dependence of heparin binding activity on differences in T3 disulfide structure, i.e., those structures that do not contain an S–S bridge have no binding activity [13]. Also, the dependence of the binding activity on the C-terminal six amino acid residues was not seen in the LPS study using the synthetic peptide. Experiments are in progress using CE and ACE to evaluate these issues with respect to the natural glycopeptide.

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References

[1] J.W. Jorgenson, K.D. Lukacs, Science 222 (1983) 266.
[2] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[3] Y.-H. Chu, G.M. Whitesides, J. Org. Chem. 57 (1992) 3524.
[4] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck, G.M. Whitesides, J. Med. Chem. 35 (1992) 2915.
[5] Y.-H. Chu, L.Z. Avila, J. Gao, G.M. Whitesides, Acc. Chem. Res. 28 (1995) 461.
[6] N.H.H. Heegaard, in S. Harding, P.Z. Chowdhry (Editors), Protein–Ligand Interactions — A Practical Approach, Oxford University Press, Oxford, 1999, in press.
[7] N.H.H. Heegaard, K. Shimura, in: P. Lundahl, E. Greijer, A. Lundqvist (Eds.), Quantitative Analysis of Biospecific Interactions, Harwood, Amsterdam, 1998, p. 15.
[8] N.H.H. Heegaard, Electrophoresis 19 (1998) 442.
[9] N.H.H. Heegaard, P.M.H. Heegaard, P. Roepstorff, F.A. Robey, Eur. J. Biochem. 239 (1996) 850.
[10] C.J.C. de Haas, M.E. van der Tol, K.P.M. Van Kessel, J.A.G. Van Strijp, J. Immunol. 161 (1998) 3607.
[11] M.B. Pepys, T.W. Rademacher, S. Amatayakul-Chantler, P. Williams, G.E. Noble, W.L. Hutchinson, P.N. Hawkins, S.R. Nelson, J.R. Gallimore, J. Herbert, T. Hutton, R.A. Dwek, Proc. Natl. Acad. Sci. USA 91 (1994) 5602.
[12] N.H.H. Heegaard, F.A. Robey, Anal. Chem. 64 (1992) 2479.
[13] N.H.H. Heegaard, Electrophoresis 19 (1998) 442.
[14] R.A. Copeland, Methods for Protein Analysis, Chapman and Hall, 1994.
[15] R.J. Beynon, J.S. Bond (Eds.), Proteolytic Enzymes, IRL, Oxford University Press, Oxford, 1990.
[16] J. Montreuil, Biochem. Soc. Trans. 11 (1983) 134.
[17] S. Dhawan, R.L. Fields, F.A. Robey, Biochem. Biophys. Res. Commun. 171 (1990) 1284.