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# Characterization of Multimodal Hydrophobic Interaction Chromatography Media Useful for Isolation of Green Fluorescent Proteins With Small Structural Differences

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**Hydrophobic interaction chromatography (HIC) has been developed as a powerful technique for separating and purifying proteins. In this study, we have characterized the ability of new multimodal pH-HIC media to resolve proteins with only small differences in their primary structures. This was done by determining the retention times of different green fluorescent protein (GFP) mutants prepared from *Escherichia coli* extracts. The mutants, modified with single or double hydrophobic amino acid substitutions in two positions, N212 and T230, could be resolved successfully, up to 2.1 column volumes in retention difference for single substitutions and 2.6 column volumes for double substitutions, at two pH and on two media with varying ligand density. The retention times also correlated well with calculated theoretical retentions ($R^2 = 0.91$) using a hydrophobic descriptor. This medium can therefore be very useful in a final polishing step during purification and the protein library prepared represents a good screening set in validating and characterizing new future media due to the accessible, but yet, extremely small differences in protein structure. Copyright © 2008 John Wiley & Sons, Ltd.**

**Keywords:** green fluorescent protein; hydrophobic interaction chromatography; tyrosine tag; pH-responsive; polymer ligand

## INTRODUCTION

The demands for high-level purity of target proteins are generally rapidly increasing, not only for therapeutic applications that require protein homogeneity, but also for industrial processes where increased purity requirements often are encountered. During cellular transcription and translation some errors inevitably occur (Valente and Kinzy, 2003), producing the desired protein as a mixture of molecules with different minor but undesirable modifications. Both basic biomedical research and applied biotechnological process technology are thus highly dependent on analytical methods that ensure the purity of target proteins; this includes requirements for the development of optimized separation materials for proteins. The generation of chromatographic media that are able to resolve minor differences in structure is essential for the effectiveness of analytical methods or in a final polishing step during purification.

Hydrophobic interaction chromatography (HIC) has gained increased attention as an efficient purification procedure in downstream processes due to its advantages in both separation and scale-up (Graumann and Ebenbichler, 2005). HIC separates proteins on the basis of hydrophobic and related interactions between immobilized hydrophobic ligands and non-polar surface-exposed regions (patches) on biomolecules. For instance, HIC separations have been explored for separating different lysozyme isozymes (Fausnaugh and Regnier, 1986). Differences in only a few hydrophilic or charged residues substantially altered the retention times on a phenyl-based HIC column. Furthermore, the separation of proteins carrying hydrophobic fusion tags has been examined. The tags, containing tryptophan or isoleucine residues, were separated on HIC columns (Hassinen *et al*., 1994), giving longer retention times for the hydrophobically modified fusion proteins.

One of the most significant drawbacks related to HIC is that some target proteins may become denatured during the process. For example, the frequently used high salt concentration buffers required for HIC may be harmful for sensitive target polypeptides, which demand for alternative ways to control binding. Previously synthesized HIC media (Yon, 1972) often showed mixed ionic and hydrophobic character due to charged linking chemistries (Wilchek and Miron, 1976).

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**Abbreviations used:** *HIC, hydrophobic interaction chromatography; GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactoside; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; QSPR, quantitative structure property relationship; SASA, solvent accessible surface area; AMBN, 2,2-azobis(2-methylbutyronitrile).*

The present study characterizes a polymer ligand HIC medium that exhibits pH-responsiveness, offering the possibility to control protein–matrix interactions under less denaturing conditions. Several factors influence the selectivity of separations in HIC systems, such as the stationary phase resin, type of salt, salt concentration, and buffer pH. Despite several efforts to improve our understanding of these operating parameters on HIC separations, the selection of appropriate chromatographic conditions for the separation of complex biological mixtures remains a challenge. Models that predict the chromatographic behavior of proteins in HIC have been widely studied and described (Lienqueo and Mahn, 2005). Even the behavior of proteins with unknown three-dimensional structure has been predicted in HIC (Salgado *et al.*, 2005a,b). Here, a set of descriptors, (Malmquist *et al.*, 2006), describing the three-dimensional structure of proteins was examined, and tested for their use in quantitative structure property relationship (QSPR) modeling of chromatographic data.

Green fluorescent protein (GFP), originally isolated from *Aequorea victoria* (Shimomura *et al.*, 1962), was chosen as the target protein to characterize our developed HIC media. It is a highly stable protein possessing a tightly packed "$\beta$-can" tertiary structure. Mature GFP is able to emit intense and stable fluorescence without any co-factors in many different organisms rendering GFPs ideal for many applications in molecular biology and biotechnology.

Different properties can be introduced genetically into a target protein to facilitate its purification and isolation. Several affinity tags for this purpose are currently available, ranging from short peptide sequences including polyhistidine tags in which the interaction between histidine residues and divalent metal ions are utilized (Ueda *et al.*, 2003). Target proteins fused with short hydrophobic peptide tags have been explored successfully in bioseparation because additional hydrophobic residues have a strong effect on the total hydrophobicity of the tagged protein (Rodenbrock *et al.*, 2001; Fexby and Bülow, 2002). The most commonly used are tryptophan-containing tags (Berggren *et al.*, 2000). However, other hydrophobic peptide tags have been examined, including polyphenylalanines (Persson *et al.*, 1988), polyisoleucines (Hassinen *et al.*, 1994), and polytyrosines (Fexby *et al.*, 2004). In this study, aqueous solutions of the engineered variant of GFP, GFPuv (Crameri *et al.*, 1996), and mutants of GFPuv harboring single or double amino acid substitutions to more hydrophobic tyrosine and isoleucine residues have been used for the characterization of the media, allowing resolution of such small differences.

## MATERIALS AND METHODS

### Biological materials and chemicals

*Escherichia coli* strain TG1 (*supE thi-1* $\Delta$(*lac-proAB*) $\Delta$(*mcrB-hsdSM*)*5* ($r_K^-$ $m_K^-$) [*F' traD36 proAB lacI*$^q$*Z*$\Delta$*M15*]) was used as host in all experiments and plasmid pTrc99a (GE Healthcare, Uppsala, Sweden) was used as expression vector. Construction of plasmid pTGFPuv, expressing the *gfpuv* gene, has been described elsewhere (Fexby *et al.*, 2004). Restriction endonuclease *Dpn*I and *pfu*Ultra DNA polymerase were obtained from Stratagene (La Jolla, USA), nucleotides from Roche (Mannheim, Germany) and the plasmid purification kit from Qiagen (Basel, Switzerland). All other chemicals were of analytical grade and are commercially available.

### Construction of GFPuv variants

The *gfpuv* gene was amplified by using plasmid pTGFPuv as template and complementary primers carrying mismatches (bold) for each modification (MWG Biotech AG, Germany) (Table 1). Except for the mismatches, the sense primers are identical to the coding strand of the *gfpuv* gene. After digestion with *Dpn*I, the newly constructed plasmids were used to transform competent *E. coli* TG1 cells. The cells were spread over Luria-Bertani (LB) agar plates containing 100 $\mu$g/ml ampicillin and 1 mM IPTG, and colonies showing fluorescence under UV light were selected and sequenced by using BigDye-terminators version 3.0 from Applied Biosystems (Warrington, UK) according to the supplier's instructions. The results were analyzed on an ABI 3100 Genetic analyzer (BM Labbet, Lund, Sweden). The procedure was repeated for the construction of proteins carrying several modifications. Proteins Y3P2, YP3, and Y3 have been constructed earlier (Fexby *et al.*, 2004).

### Protein expression

Cells were grown in modified LB medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 100 $\mu$g/ml ampicillin. A shake flask containing 200 ml of modified LB medium and 100 $\mu$g/ml ampicillin was inoculated with 1 ml of an overnight culture, and gene expression was induced with 1.0 mM isopropyl-$\beta$-D-thiogalactoside (IPTG) when $OD_{600}$ reached 0.6. The cells were grown overnight, harvested in late logarithmic phase (5000 g, 5 min) and resuspended in 5 ml of 50 mM sodium phosphate buffer, pH 7.0. The cell slurry was sonicated and centrifuged (27 000*g*, 15 min).

### Pre-purification of proteins

The supernatants were subsequently heated at 70°C for 10 min and centrifuged (27 000*g*, 15 min). Contaminating proteins were removed by precipitation with 1.5 M ammonium sulphate $(NH_4)_2SO_4$. After centrifugation (27 000*g*, 15 min), GFPuv constructs were precipitated from the supernatant with 2.8 M $(NH_4)_2SO_4$. Centrifuged pellets (27 000*g*, 15 min) were resuspended in 50 mM sodium phosphate buffer, pH 7.0, and dialyzed (MWCO: 3500) in the same buffer overnight. Samples were heat-treated once again at 70°C for 10 min and centrifuged (27 000*g*, 15 min). The supernatants were stored at −20°C until further use. The purity of the proteins was routinely checked by

**Table 1.** Oligonucleotides used in the amplification of the *gfpuv* gene

| | |
|---|---|
| Sense primer N212Y | 5-CCTTTCGAAAGATCCC**TAC**GAAAAGCGTGACCACA-3 |
| Sense primer N212I | 5-CCTTTCGAAAGATCCC**ATC**GAAAAGCGTGACCACA-3 |
| Sense primer T230Y | 5-GTAACTGCTGCTGGGATT**TAC**CATGGCATGGATGAGCTC-3 |
| Sense primer T230I | 5-AACTGCTGCTGGGATT**ATA**CATGGCATGGATGAGC-3 |

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15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using a Tris-glycine, pH 8.3, discontinuous buffer system (Laemmli, 1970). Purified protein extracts were assayed for total protein concentrations using the Bradford protein assay (Bradford, 1976). The steady-state fluorescence measurements of the protein samples were performed on a fluorometer (Photon Technology International, West Sussex, UK). The excitation wavelength was set to 400 nm in all experiments, and emission was monitored at 508 nm.

### Chromatographic conditions

The chromatography media were prepared as described previously, (Van Alstine *et al*., 2006) by using AMBN at two concentrations, 2% and 16%, as initiator yielding two media varying in ligand density (Figure 1). Chromatography experiments involving protein extracts were carried out with an ÄKTApurifier™ system controlled by UNICORN™ software (GE Healthcare, Uppsala, Sweden). The two HIC media with variation in molecular weight and ligand density, high and low substitution (GE Healthcare, Uppsala, Sweden) were packed in HR 5/5 columns to 1 ml. Binding buffers used were 0.1 M sodium phosphate, 1.5 M $(NH_4)_2SO_4$, pH 7.0 and 0.1 M sodium acetate, 1.5 M $(NH_4)_2SO_4$, pH 5.0. The columns were equilibrated for 12 column volumes (CV) with binding buffer followed by an injection of 100 μl sample containing 0.3–0.5 mg protein. A gradient elution (20 CV) from 1.5 to 0 M $(NH_4)_2SO_4$ at a constant flow rate of 1 ml/min was applied and protein concentrations were detected at 215 or 280 nm. Fractions were collected and checked for fluorescence under UV light.

### QSPR modeling

The high-resolution structure of a GFPuv variant (PDB: 1qyo) was used as a starting point for the modeling. The GFPuv constructs were modeled by mutating this variant to the appropriate sequence and by giving the additional *N*-terminal segment a random secondary structure. Fifty-eight descriptors, which describe protein surface properties, were calculated for the GFPuv models using the proprietary program SCARP version 2.6 (Malmquist *et al*., 2006). The data was used for QSPR modeling with the program 'The Unscrambler' (Camo, 2005) version 9.2. The default PLS1 method was used for partial least squares regression. Both descriptors and response values were auto scaled, that is, centered by subtracting the mean value and divided by the standard deviation, during the calculations. The automatic suggestion from the program to exclude for the data set invariant descriptors in the QSPR calculations was followed. After each regression, a new calculation was performed by using only those descriptors which were shown to be significant in the previous run until the model was based on significant descriptors only.

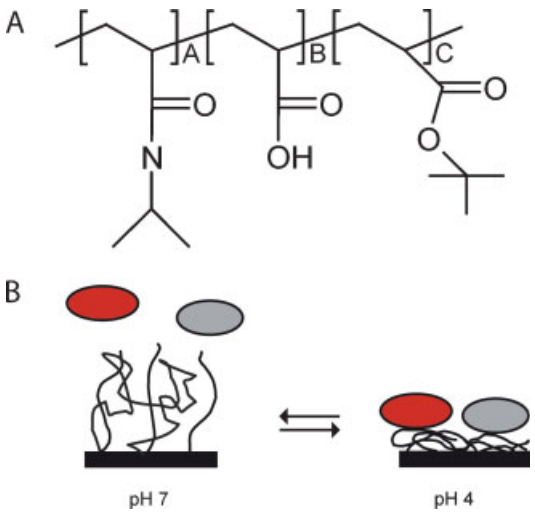


**Figure 1.** (A) The general formula for the responsive polymer coating developed to have pH-HIC responsiveness over the acidic pH range. It is composed of a self-associating group, A, with some charge as well as hydrophobic character, a group, B, added to control pH responsiveness, and group C to improve HIC functionality. (B) Schematic of the behavior of the media with varying pH.

## RESULTS AND DISCUSSION

### pH-responsive media

The HIC media carry three functional groups, which gives the option to combine both hydrophobic interactions with a pH-responsiveness during the isolation of proteins (Figure 1). The hydrophobic interactions can thereby be tuned by the pH of the mobile phase and/or the salt concentration employed. The interactions of the target molecule with the matrix can hence be increased when more acidic conditions are utilized. The matrix is provided with a flexible polymer surface coating, which changes conformation relative to the target compound during the adsorption and elution processes. This conformational change of the polymers is based on polymer self-association and disassociation with the matrix when changing pH. At lower pH the carboxyl group (group B, Figure 1), which contributes to the pH-responsiveness, associates with the matrix-exposing group C with more hydrophobic character (tert-butyl) and the electrostatic interactions between protein and media become less prominent. The degree of hydrophobicity can therefore be controlled, with more hydrophobic characteristics at lower pH.

### Protein library and expression

The influence of tyrosine and isoleucine residues on the retention on the HIC media was investigated by eight GFPuv variants that were constructed with modifications in a single or double position internally in the protein, positioned at N212 and T230 (Figure 2A, Table 2). The positions for substitution were selected for minimal disturbance and modifications of the original protein, yielding variants with similar structures, hence minimizing the structural influence on the hydrophobic properties and on protein isolation. Furthermore, existing asparagine and threonine in the native GFPuv are believed not to be involved in any existing hydrogen bonds. Both positions are also surface exposed with side-chains pointing outwards from the protein. These variants were compared with previously studied hydrophobic-tagged GFPuv variants. The proteins were expressed intracellulary in *E. coli* under IPTG induction. SDS–PAGE of purified extract revealed that all proteins were well expressed (Figure 2B). GFPs carrying tags YP3 and Y3P2 were as previously reported more ubiquitously expressed than the native protein (Fexby *et al*., 2004). The amount of corresponding mRNAs was found to correlate with the amount of proteins expressed which suggested that changes in mRNA stability can affect the expression levels. Additionally, all variants carrying substitutions

  

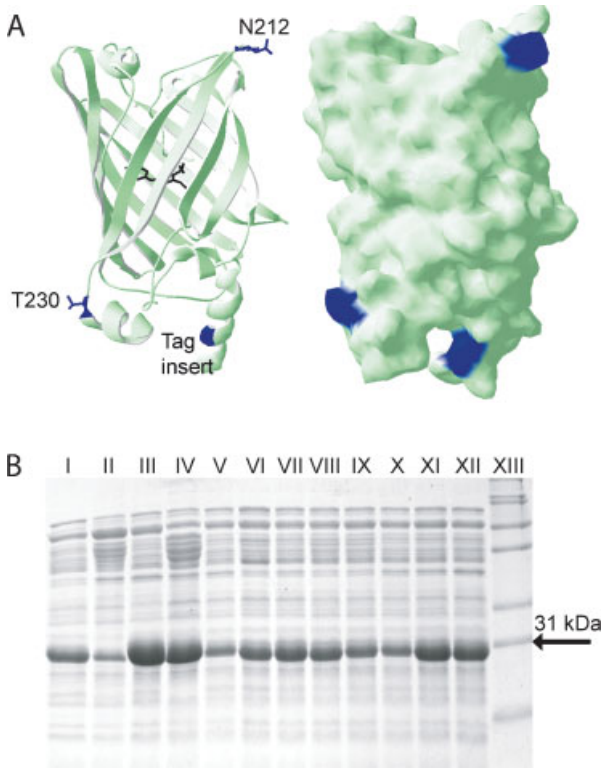


**Figure 2.** (A) Structure of GFPuv with positions of modifications shown in dark blue and the fluorophore in black in the center of the protein. Position N212 is located in a readily accessible loop opposite to the protein terminals, and T230 is located close to the carboxyl terminal. Both positions were subjected to site-specific mutagenesis. Also shown is the position of the tag insert at the amino terminal described previously. (B) Expression of purified GFPuv variants shown on a 15% SDS–PAGE gel. I = GFPuv, II = Y3, III = YP3, IV = Y3P2, V = NI, VI = TY, VII = NY, VIII = TI, IX = NITI, X = NITY, XI = TYNY, XII = TINY.

for tyrosine residues were more expressed than substitutions for isoleucine residues, indicating a clear effect of tyrosine residues on the expression level probably due to mRNA stabilization or alternatively to stabilization of the protein during purification.

**Table 2.** Amino terminal sequences of the GFPuv variants constructed

| Variant | Amino acid sequence (modifications) |
|---|---|
| GFPuv | MEFELGT-GFPuv |
| NI | MEFELGT-GFPuv (N212I) |
| TY | MEFELGT-GFPuv (T230Y) |
| NY | MEFELGT-GFPuv (N212Y) |
| TI | MEFELGT-GFPuv (T230I) |
| NITI | MEFELGT-GFPuv (N212I, T230I) |
| NITY | MEFELGT-GFPuv (N212I, T230Y) |
| TYNY | MEFELGT-GFPuv (T230Y, N212Y) |
| TINY | MEFELGT-GFPuv (T230I, N212Y) |
| Y3 | MEFELYYYASGT-GFPuv |
| YP3 | MEFELYPYPYPASGT-GFPuv |
| Y3P2 | MEFELYYYPPASGT-GFPuv |

Modifications within the protein are shown in parenthesis.

### Chromatography experiments

Initially, separations of individual proteins were made on two media at two pH values, 5.0 and 7.0, with the proteins eluted in the first half of the gradient. Commercial HIC media, such as phenyl sepharose, bind the hydrophobic GFPuv extremely strongly and elution is not possible or the proteins are eluted at the very end of the gradient with 0 M $(NH_4)_2SO_4$ (data not shown). Similar results have previously been described (McRae *et al*., 2005), where addition of isopropanol was needed for elution to occur from a commercial media. This means that the media described here is more applicable for bioseparations using HIC, especially for hydrophobic proteins. The retention difference normalized for GFPuv, $\Delta Z$, was calculated for each of the GFPuv variants (Figure 3). The retention appears to follow the theoretical hydrophobicity with variants carrying hydrophobic tags having the strongest retention, followed by the GFPs carrying double and single substitutions, respectively. From a polishing point of view, the mutants can be divided into three groups depending on the number of amino acid substitutions (1) NI, TI, TY, NY, (2) TINI, TYNI, TINY, TYNY, and (3) YP3, Y3, Y3P2. Furthermore, variants with tyrosine residues showed stronger retention than those carrying isoleucine residues. The results suggest that the polarity of the tyrosine residue is important for the binding to the HIC media. The influence of tyrosine residues on $\Delta Z$ can clearly be seen by studying NY, TYNY which have significantly higher $\Delta Z$ compared to the isoleucine-containing mutants in this group, NI and TINI. NY even shows higher $\Delta Z$ than some mutants carrying double substitutions. In addition, TINI, in some cases, results in lower $\Delta Z$ than mutants with a single substitution.

There is an appreciable difference in $\Delta Z$ with varying pH. At pH 5.0, $\Delta Z$ is larger than at pH 7.0. This may be due to the increased hydrophobic effect of the media at pH around 5.0 and the proximity to the pI of GFPuv (pI = 5.57), which decreases the effects of charged residues. The $\Delta Z$ appears to be larger at neutral pH for the first group carrying single modifications, indicating that other interactions may occur in the column creating a multi-modal pH-HIC media.

The molecular weight of the polymer ligand (high and low substitution) contributes to the difference in $\Delta Z$ between the columns (Figure 3). Higher molecular weight increases the retention time for hydrophobic compounds, resulting in larger $\Delta Z$ on the high substitution column than on the one with low substitution. The conformational changes of the media at lower pH also change the interactions. This effect is most noticeable for the high substituted media with larger increase of $\Delta Z$ by lowering pH. One possibility is that the polymer ligand associate more with ligands in the vicinity and with the matrix thus creating a more densely packed and firm coating with lower pH with higher hydrophobic properties, compared to the more free and flexible ligands in the low substituted media with less interactions between the ligands themselves.

Retention due to the position of the amino acid modification is also a factor to consider. Substitutions at N212 give a higher retention than substitutions at T230, an indication that the loop of N212 is more accessible and an interesting point for attachment of future tags useful for protein purification. T230 is located in the more flexible carboxyl terminal, and can therefore be turned inwards into the protein barrel, thus decreasing its influence on the hydrophobic properties.

After analyzing the retention values of the individual proteins, mixtures of native and mutant GFPuvs were applied to the low

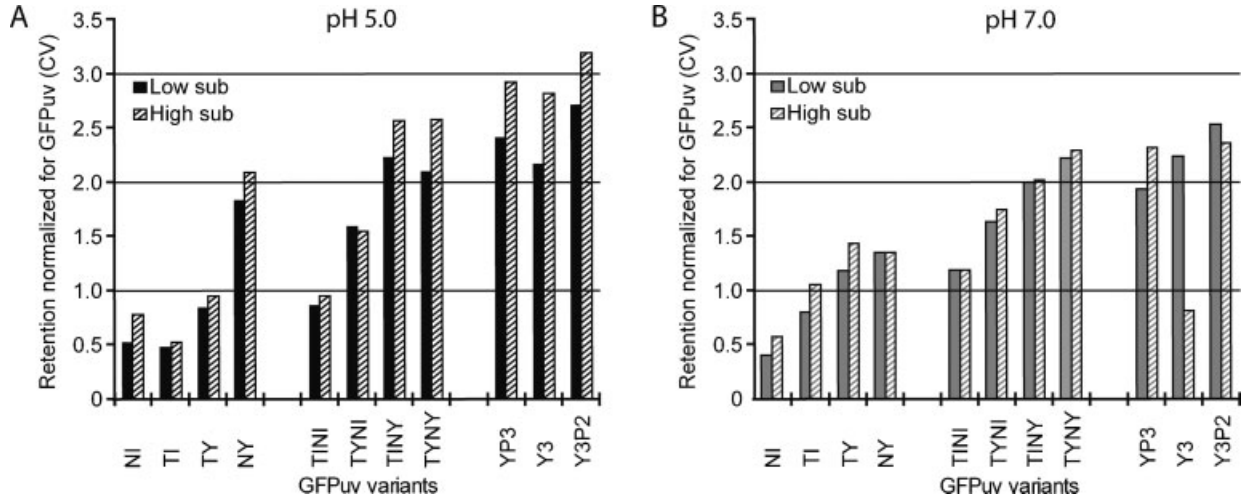


**Figure 3.** Retention of the GFPuv variants normalized for GFPuv on the two columns at pH 5.0 (A) and pH 7.0 (B) grouped in single amino acid substitutions, double substitutions, and amino terminal tags.

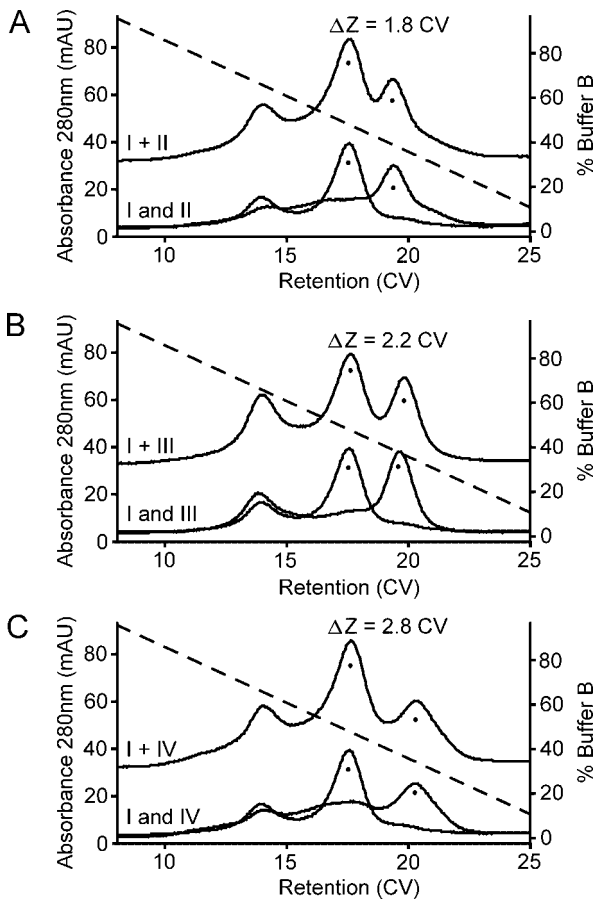


**Figure 4.** Individual chromatograms of GFPuv samples compared to chromatograms of mixed samples containing native and mutant GFPuv from each group that had the largest $\Delta Z$ at pH 5.0 on the high substitution media, that is, NY, TYNY, and Y3P2. (A) I = GFPuv, II = NY (B) I = GFPuv, III = TYNY (C) I = GFPuv, IV = Y3P2. The fluorescent peaks are marked with a dot and the dotted line represents the concentration of elution buffer. A contaminant peak is eluted before the GFPuv fluorescent peaks in all experiments.

substitution column at pH 5.0 to assess separations of mixed GFPuv variants in the same sample (Figure 4). Two well-separated peaks were observed, which were in good correlation with $\Delta Z$ observed in single protein sample experiments. The $\Delta Z$ of the three mixtures analyzed was increased with the number of hydrophobic substitutions. For group 1, $\Delta Z = 1.8$ CV, group 2, $\Delta Z = 2.2$ CV, and group 3 $\Delta Z = 2.8$ CV. The media are therefore able to separate proteins carrying single amino acid substitutions in the same sample, not only the extreme hydrophobic resides, such as tryptophan, but also with the less hydrophobic isoleucine and the hydrophilic tyrosine residue.

### QSPR modeling

The experimentally observed retention data were compared with corresponding results obtained from the modeling. At lower pH, the acidic groups on both the protein surface and the media become protonated and thus uncharged. Therefore the contribution of charge at pH 5.0 is expected to be smaller as

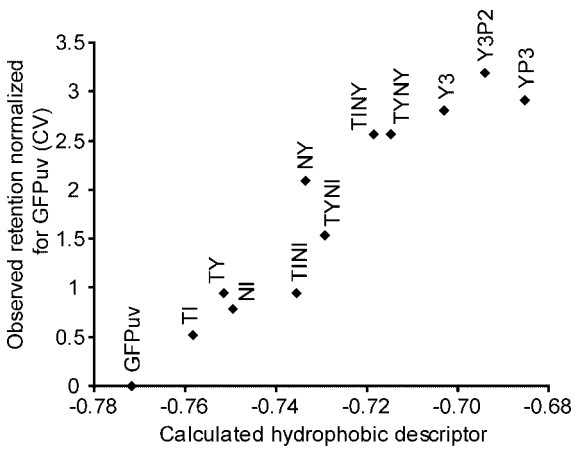


**Figure 5.** Observed retention time at pH 5.0 versus the calculated hydrophobic descriptor Hydrophob 3Norm.

  

compared to pH 7.0. This was confirmed by PLS-regression of the pH 5.0 data set which showed that the charged descriptors lacked significance and that the only significant descriptor was a hydrophobic descriptor (Hydrophob_3Norm). The observed retention times for the data set on the high substitution media at pH 5.0 was plotted against the significant descriptor for every variant (Figure 5). The correlation ($R^2 = 0.91$) suggests that hydrophobicity is relevant for the $\Delta Z$ of the GFPuv variants on the HIC media at pH 5.0. The tyrosine side chain has larger solvent accessible surface area (SASA) than the isoleucine side chain yielding higher hydrophobicity according to the hydrophobic descriptor, which also is reflected in the retention data.

## CONCLUSION

Multimodal HIC media, where the degree of hydrophobicity can be controlled by variation of pH, has proved to be superior to conventional HIC media, for example, phenyl or hexyl sepharose, for protein separations. Since small differences in protein structure can be resolved, the media could be particularly useful for proteomics applications.

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