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Review

Recent progresses in protein-based chiral stationary phases for enantioseparations in liquid chromatography☆

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ABSTRACT

Chiral stationary phases (CSPs) based on proteins or glycoproteins have been developed for the enantioseparations of various compounds. In 2001, a review article [J. Haginaka, J. Chromatogr. A, 906 (2001) 253] dealing with CSPs based on proteins and glycoproteins was published. After that serum albumin from other species, penicillin G-acylase, antibodies, fatty acid binding protein and streptavidin were newly introduced as the chiral selectors in CSPs. This review article deals with recent progresses in CSPs based on protein or glycoproteins in LC after 2001, focusing on their enantioselective properties and chiral recognition mechanisms.

Contents

1. Introduction 13
2. Recent progresses in CSPs based on proteins or glycoproteins 13
   2.1. Human serum albumin 13
   2.2. α_1-Acid glycoprotein 13
   2.3. Ovoglycoprotein (chicken α_1-acid glycoprotein) 14
   2.4. Glucoamylase 16
   2.5. Penicillin G-acylase 16
   2.6. Antibodies 17
   2.7. Other proteins 18
3. Conclusions 18
   Acknowledgements 18
   References 18

Abbreviations: ABA, abscisic acid; AGP, α_1-acid glycoprotein; cAGP, chicken α_1-acid glycoprotein; BSA, bovine serum albumin; CD, circular dichroism; CSP, chiral stationary phase; DMOA,*N*,*N*-dimethyl-*n*-octylamine; DSC, *N*,*N*′-disuccinimidylcarbonate; EDMA, ethylene glycol dimethacrylate; Fab, antigen binding fragment; FAGP, fatty acid binding protein; GMA, glycidyl methacrylate; HSA, human serum albumin; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; OGCHI, ovoglycoprotein from chicken egg whites; OMCHI, ovomucoid from chicken egg whites; ORM, orosomucoid; ORM 1, F1–S variants of orosomucoid; ORM 2, A variants of orosomucoid; PGA, penicillin G-acylase; SIC, succinimidyl iodoacetate; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

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1. Introduction

A lot of chiral stationary phases (CSPs) are available for enatioseparations of various compounds in LC. Among those, protein-based CSPs have the enantioselectivity for a wide range of compounds because of a multiple binding site(s) on the surface of a chiral selector and/or a multiple binding interaction(s) between a chiral selector and ligand [1–3]. In 2001, a review article dealing with CSPs based on proteins and glycoproteins was published [3]. Serum albumin from other species [4], penicillin G-acylase (PGA) [5], antibodies [6], fatty acid binding protein (FABP) [7] and streptavidin [8] were newly introduced as the chiral selectors in CSPs. One of the major drawbacks of conventional protein-based CSPs is that the elution order of an enantiomer is unpredictable and dependent on an immobilized protein. Antibodies are glycoproteins, which are produced by the immune system of vertebrates in response to antigens, invading pathogenic microorganisms or non-self biological compounds [9]. Antibodies are stereoselective and can recognize enantiomeric compounds [9]. Recently, antibody-based CSPs have been introduced [6,9], where the elution order of an enantiomer is predictable. This review article mainly deals with recent progresses in CSPs based on proteins or glycoproteins in LC after 2001, focusing on their enantioselective properties and chiral recognition mechanisms.

2. Recent progresses in CSPs based on proteins or glycoproteins

2.1. Human serum albumin

Hage and co-workers bound human serum albumin (HSA) to polymer monoliths based on glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) [10]. Furthermore, diol-silica monoliths and polymer monoliths based on GMA and EDMA were oxidized by using periodic acid to give aldehyde groups, and allowed an amine group of HSA to react with aldehyde groups to form a Shiff base followed by reductive amination [11]. The surface coverage of HSA in the silica monolith was similar to values obtained with silica particles and a GMA/EDMA monolith. Because of its higher surface area, the silica monolith contained 1.3–2.2 times more immobilized HSA per unit volume when compared to silica particles or a GMA/EDMA monolith. Therefore, the HSA silica monolith gave higher retention, and higher or comparable resolution and efficiency when compared to HSA columns that contained silica particles or a GMA/EDMA monolith. It was concluded that silica monoliths can be valuable alternatives to silica particles or GMA/EDMA monoliths when used with immobilized HSA as a CSP [11].

Furthermore, HSA was immobilized via its sulfhydryl groups [12]. Amino-silica was activated by succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) followed by reaction with a sulfhydryl group(s) of the protein [12]. Similarly, amino-silica was activated by succinimidyl iodoacetate (SIC) for reaction with a sulfhydryl group [12]. Maleimide-activated silica (the SMCC method) or iodoacetyl-activated silica (the SIA method) was used for these methods as illustrated in Fig. 1. It was found that the SMCC and SIA methods gave HSA-based CSPs with comparable or improved activity and stability, compared to those made by the Schiff base method. Fig. 2 shows enantioseparations of tryptophan, warfarin and ibuprofen on HSA columns prepared by the SMCC and SIA methods [12]. The SMCC method gave the best overall behavior.

HSA is closely related proteins with serum albumins from other mammalian species. However, the binding stereoselectivity of the

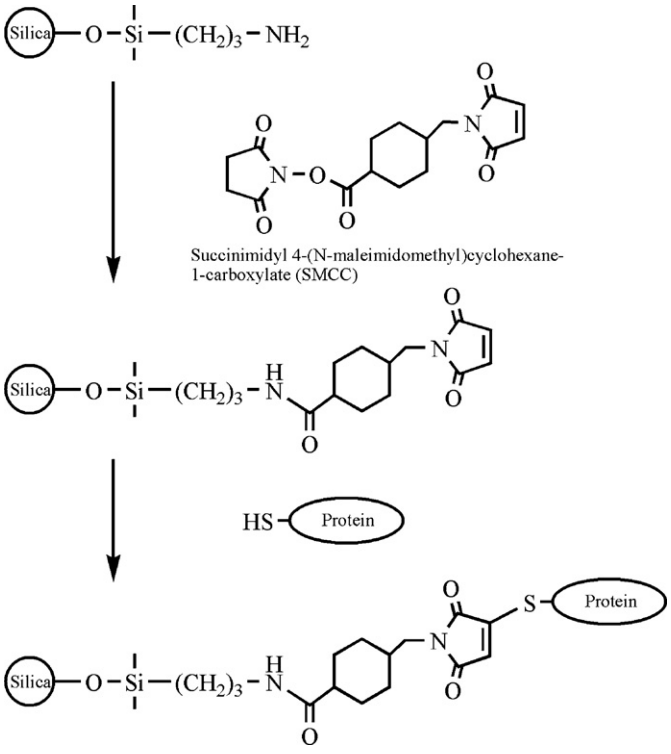

Fig. 1. Synthesis scheme for the preparation of protein-based CSPs via the sulfhydryl group of the protein.

2,3-benzodiazepine, tofisopam, in human, is opposite to that in all other species (bovine, dog, horse, pig, rabbit and rat). In the binding of 1,4-benzodiazepines, dog albumin is very similar to HSA [4].

Stereoselective binding characteristics of HSA have been thoroughly examined: stereoselective binding of drugs on HSA occurs principally at two major binding sites; warfarin-azapropazone site (site I) and indole-benzodiazepine site (site II) (see Fig. 3). He and Carter determined the three-dimensional structure of HSA, which shows that the binding sites I and II are located in hydrophobic cavities in subdomains IIA and IIIA, respectively [13]. In addition, the crystal structure of HSA-myristate complexed with the (*R*)- and (*S*)-warfarin was determined [14]. The structures confirm that warfarin binds to subdomain IIA in the presence of fatty acids and reveal the molecular details of the protein–drug interaction. The two enantiomers of warfarin adopt very similar conformations when bound to the protein and make many of the same specific contacts with amino acid side chains at the binding site, thus accounting for the relative lack of stereospecificity of the HSA-warfarin interaction [14]. Furthermore, Chuang and Otagiri [15] suggested that the stereoselective binding site might exist at the interface of the subdomains.

2.2. α_1-Acid glycoprotein

Hage and co-workers bound α_1-acid glycoprotein (AGP, orosomucoid (ORM)), which is a member of the lipocalin family, via its carbohydrate chains after periodate oxidation to hydrazide-activated supports [16,17]. Silica particles, silica monoliths and polymer monoliths based on GMA and EDMA were used as the support [17]. The surface coverage of AGP in the silica monolith was 18% higher than that obtained with silica particles and 61% higher than that with a GMA/EDMA monolith. The higher surface area of the silica monolith gave materials that contained 1.5–3.6 times more immobilized protein per unit volume when compared

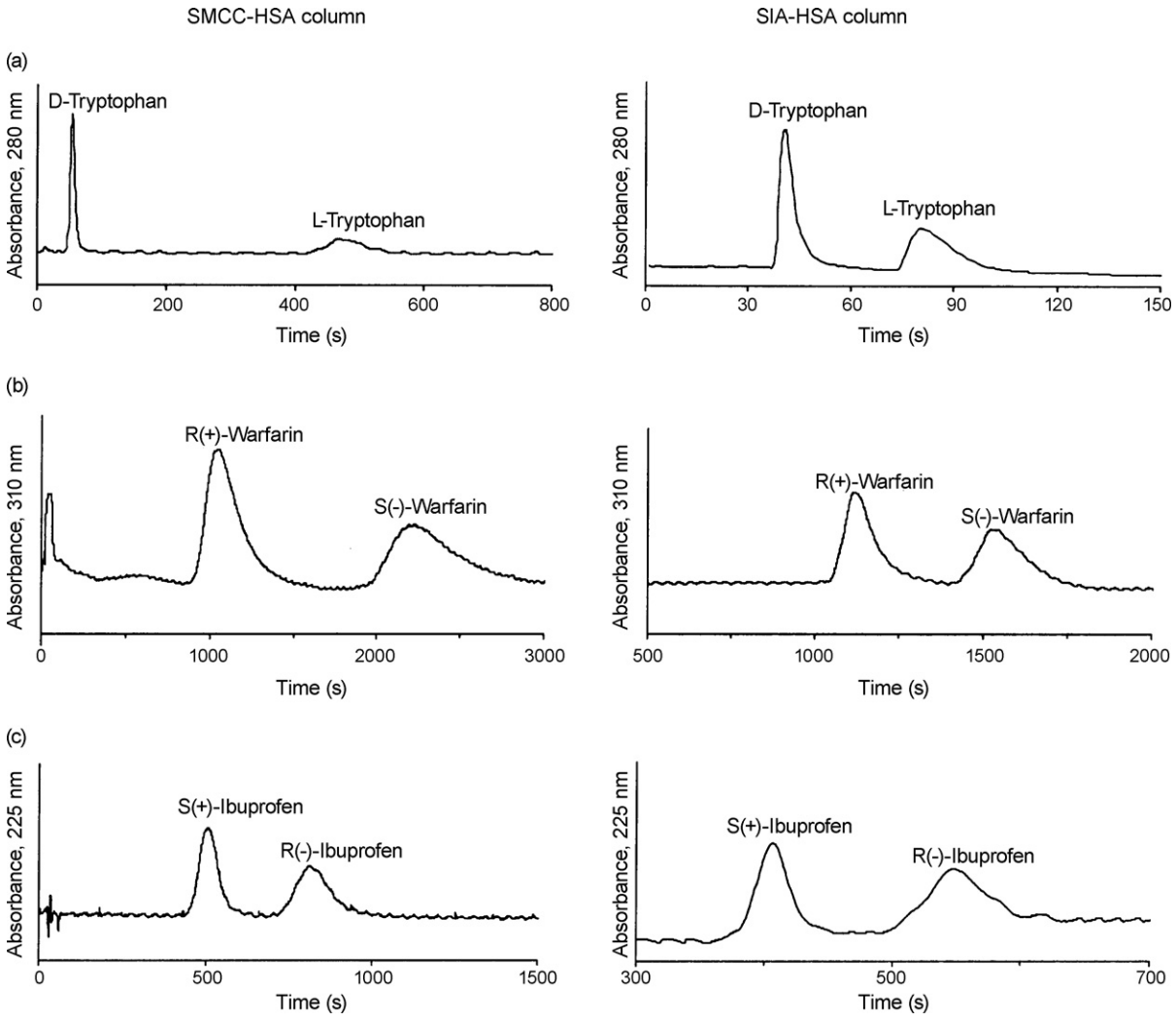

Fig. 2. Enantioseparations of (a) D/L-tryptophan, (b) *R*/*S*-warfarin and (c) *R*/*S*-ibuprofen on HSA columns prepared by the SMCC and SIA methods. The HPLC conditions were as follows: sample concentration, 20 μM tryptophan, ibuprofen or warfarin; sample volume, 20 μL; mobile phase for tryptophan, pH 7.4, 0.067 M potassium phosphate buffer; mobile phase for ibuprofen, pH 7.0, 0.067 M potassium phosphate buffer containing 8% 2-propanol and 1 mM octanoic acid; mobile phase for warfarin, pH 7.0, 0.067 M potassium phosphate buffer containing 5% 2-propanol and 1 mM octanoic acid; flow rate for the SMCC HSA column, 1.5 mL/min for tryptophan, 1.0 mL/min for ibuprofen and warfarin; flow rate for the SIA HSA column, 0.3 mL/min for tryptophan and warfarin, 0.5 mL/min for ibuprofen; column size, 5 cm × 4.6 mm i.d.; temperature, 25 °C [12].

to silica particles or a GMA/EDMA monolith. The AGP silica monolith gave higher retention, and better resolution and efficiency than AGP columns containing silica particles or a GMA/EDMA monolith [17].

Besides the high heterogeneity of its glycans, the protein part of AGP has also been found to show polymorphism. The variants are encoded by two different genes: the F1 and S variants are encoded by the alleles of the same gene, while the A variant is encoded by a different gene [18]. There is a difference of at least 22 amino acid residues between the F1–S (ORM 1) and A (ORM 2) variants, while F1 and S forms differ only in a few residues. Selective binding of coumarin enantiomers (warfarin, phenprocoumon and acenocoumarol) to human AGP genetic variants was investigated. All investigated compounds bound stronger to ORM 1 than to ORM 2 [18]. ORM 1 and human native AGP preferred the binding of (*S*)-enantiomers of warfarin and acenocoumarol, while no enantioselectivity was observed in phenprocoumon binding. Furthermore, a new homology model of AGP was built and the models of ORM 1 and ORM 2 suggested that the binding cavity, including Trp122, for ORM 1 was the same with that for ORM 2, and that difference in binding to AGP genetic variants could be caused by steric factors: ORM 2 formed a smaller, more hydrophobic cavity as compared to ORM 1 [18]. Dockings to ORM 1 resulted in a much lower intermolecular energy than dockings to ORM 2, suggesting that although binding to both variants is possible, ORM 1 binding is more favorable. Energy differences between (*R*)- and (*S*)-enantiomers are not significant and show a slight preference for (*S*)-enantiomers in the case of both ORM 1 and ORM 2 [18].

Ligand-binding properties of AGP were investigated by using circular dichroism (CD) methods [19]. The induced CD spectra of drug–AGP complexes were observed with a lot of class of drugs. Results of additional CD experiments performed by using recombinant AGP mutants showed no changes in the ligand binding ability of Trp122Ala in sharp contrast with the Trp25Ala which was unable to induce extrinsic CD signal with either ligand. These findings suggest that, likely via π–π stacking mechanism, Trp25 is essentially involved in the AGP binding of drugs studied [19].

2.3. Ovoglycoprotein (chicken α_1-acid glycoprotein)

A cDNA clone encoding ovoglycoprotein from chicken egg whites (OGCHI), which is included in crude ovomucoid (OMCHI),

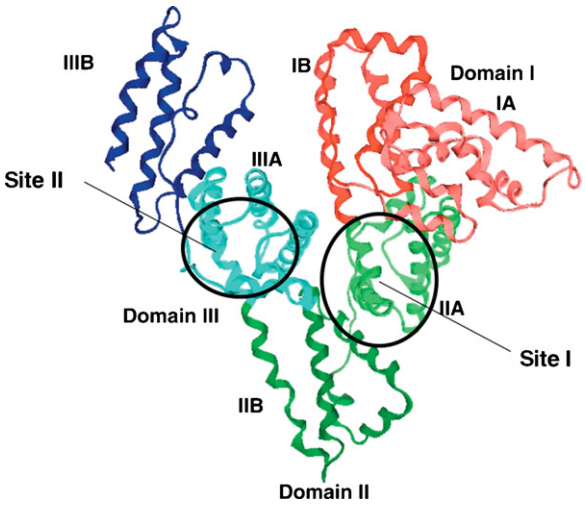

Fig. 3. Crystal structure of HSA. The subdivision of HSA into domain (I–III) and subdomains is indicated, and approximate locations of sites I and II are shown [15].

was isolated and the amino acid sequence of OGCHI was clarified [20]. OGCHI consisted of 203 amino acids including a predictable signal peptide of 20 amino acids. The mature OGCHI showed 31–32% identities to rabbit and human AGPs. Thus, OGCHI should be the chicken AGP (cAGP), a member of the lipocalin family. Furthermore, the recombinant cAGP was prepared by the *Escherichia coli* expression system, and its chiral recognition ability was confirmed by CE. Since proteins expressed in *E. coli* are not modified by any sugar moieties, this result shows that the protein domain of the cAGP is responsible for the chiral recognition [20]. cAGP consists of 183 amino acid residues and has only one Trp residue at the 26 position [20]. The Trp26 residue was modified with 2-nitrophenylsulfenyl chloride and chiral separations of neutral, acidic and basic compounds were examined on cAGP and Trp-modified cAGP columns [21]. Chiral separations of propranolol, alprenolol and oxprenolol were lost on the Trp-modified cAGP column, while chlorpheniramine, ketoprofen and benzoin were still enantioseparated on the Trp-modified cAGP column despite of lower enantioselectivity than that on the cAGP column. These results suggest that the Trp26 residue could be responsible for chiral recognition of these compounds. Competition studies using *N*,*N*-dimethyl-*n*-octylamine (DMOA) as a competitor indicated that propranolol, alprenolol and oxprenolol competed with DMOA on a single binding site near the Trp26 region and that further bindings of chlorpheniramine, ketoprofen and benzoin occurred at the secondary binding site in a non-competitive fashion with DMOA [21]. Furthermore, ligand-binding properties of cAGP were investigated by using CD methods. Analysis of the extrinsic CD spectra with the study of the Trp26-modified protein and CD displacement experiments revealed that a single Trp26 residue of cAGP conserved in the whole lipocalin family is part of the binding site, and that it is essentially involved in the ligand-binding process via π–π stack-

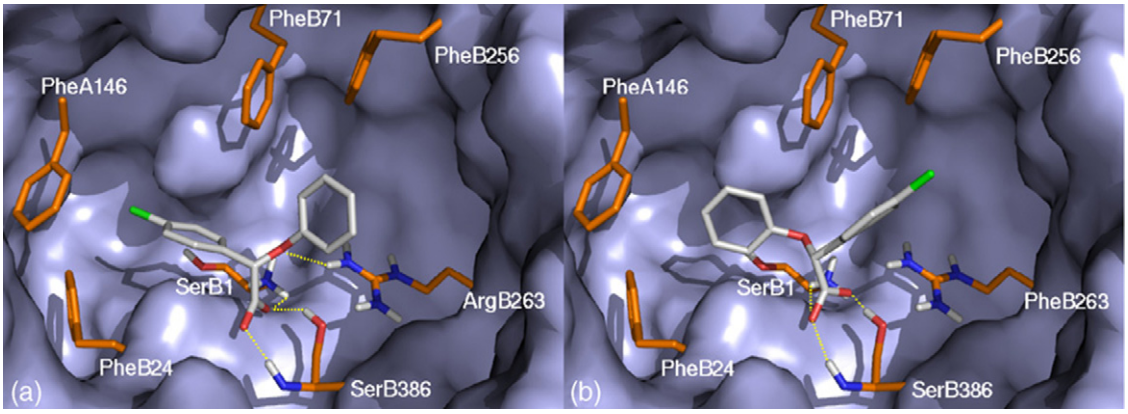

Fig. 4. Binding mode of compound (*S*)-2-(4-chlorophenyl)-2-phenoxyacetic acid (a) and (*R*)-2-(4-chlorophenyl)-2-phenoxyacetic acid (b) within PGA [31].

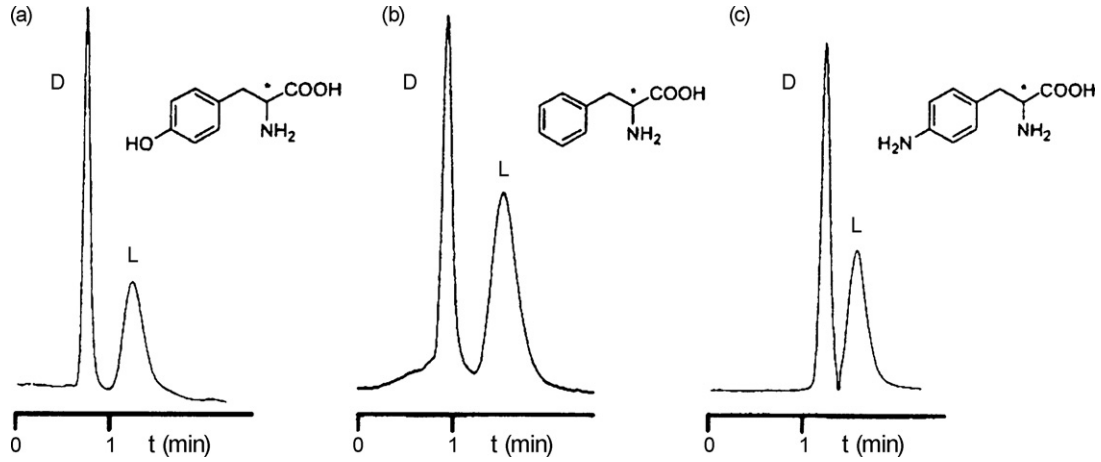

Fig. 5. Enantioseparations of (a) tyrosine, (b) phenylalanine and (c) *p*-aminophenylalanine on an anti-L-amino acid antibody column [34]. HPLC conditions: column, 250 mm × 4.6 mm i.d.; mobile phase, phosphate-buffered saline, pH 7.4; flow rates of (a and b) 4 mL/min and (c) 2 mL/min.

ing interaction resulting in the appearance of strong induced CD bands due to the non-degenerate intermolecular exciton coupling between the π–π^* transitions of the stacked indole ring–ligand chromophore [22].

2.4. Glucoamylase

CSPs based on glucoamylases G1 and G2 from *Aspergillus niger* have been introduced by Karlsson and his co-workers [23,24]. Glucoamylase G1 consists of three parts: the first 440 amino acids contain the catalytic domain, the 441–511 amino acids contain a highly *O*-glycosylated linker segment, and the 512–616 amino acids contain a C-terminal domain responsible for raw starch binding and dispensable for activity. Glucoamylase G2 consists of 512 amino acids, including the catalytic domain and linker segment without a C-terminal domain [25]. First, glucoamylase G1 was immobilized on oxidized diol-silica gels by reductive amination. The diol-silica gels with 50 nm pore-sizes gave the highest enantioselectivity and stability among the diol-silica gels tested. On this CSP, amino alcohols were separated with high separation factors and high column efficiencies up to 30,000 m^{-1}, while acidic enantiomers could not be separated [23]. Next, glucoamylase G2 was immobilized onto the same diol-silica gels [24]. Two glucoamylase-based CSPs were compared with regard to retentive and enantioselective properties for amino alcohols. The retention factors were higher using glucoamylase G2 as a chiral selector, while the enantioselectivity was similar. The higher retention factor is due to that glucoamylase G2 is more immobilized by about three times on the support, compared to the G1 enzyme [24]. Addition of acarbose, an inhibitor that binds to the catalytic domain, to the mobile phase, resulted in total loss of enantioselectivity of glucoamylase G2-based CSPs [24]. These results indicate that the catalytic domain is responsible for the chiral recognition.

The drastic increase in enantioseparation of β-blockers by increasing the content of 2-propanol in the mobile phase as well as the increase in enantioselective retention by increasing the column temperature were shown on glucoamylase G1-based CSPs [23]. Further thermodynamic study indicates that the bi-Langmuir model (containing nonselective and enantioselective sites) describes the system well [26]. The retention of (*R*)- and (*S*)-propranolol or -alprenolol at low temperatures increases with the content of 2-propanol in the mobile phase, due to the increased saturation capacity of the enantioselective sites. The retention is an enthalpy-driven process at both nonselective and enantioselective sites, whereas the enantioseparation is due to differences between the entropy changes of the two enantiomers at the enantioselective sites [26]. The enthalpy of adsorption at the nonselective sites is almost identical at the two concentrations (2.5 and 20%) of 2-propanol in the mobile phase. Enantioselective adsorption, on the other hand, is more exothermic at higher modifier content (20%). Thus, at high temperatures the retention decreases with increasing modifier content, whereas the opposite trend is the case at low temperatures [26].

2.5. Penicillin G-acylase

PGA of *E. coli* catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid [27]. PGA was immobilized via its amino and carboxy groups [5]. The former was attained using amino-silica gels activated by *N*,*N*′-disuccinimidylcarbonate (DSC) and epoxy-silica gels, and the latter was using amino-silica gels with 1-ethyl-3-(3′-dimethylaminopropyl)carbodimide and *N*-hydroxysulfosuccinimide. The best results in terms of bound amount of PGA, enzymatic activity and enantioselectivity were obtained with PGA-immobilized on epoxy-silica particles. Enantiomers of 2-arylpropionic acid derivatives such as ketoprofen, suprofen and fenoprofen, and phenoxypropionic acids such as 2-(4-phenoxyphenoxy)propionic acid and 2-(4-benzylphenoxy)propionic acid were separated on PGA-based CSPs [5]. Furthermore, PGA was immobilized on epoxy-silica monoliths [28]. Silica particle- and monolith-based CSPs were compared with their enantioselectivity [28]. The former gave the higher resolution than the latter, if considering that the amount of bound PGA was lower in the former stationary phase. The worse resolution values on the latter column are the consequence of the lower selectivity unlike the efficiency. A possible explanation could be that the higher protein coverage on the PGA monoliths determines an

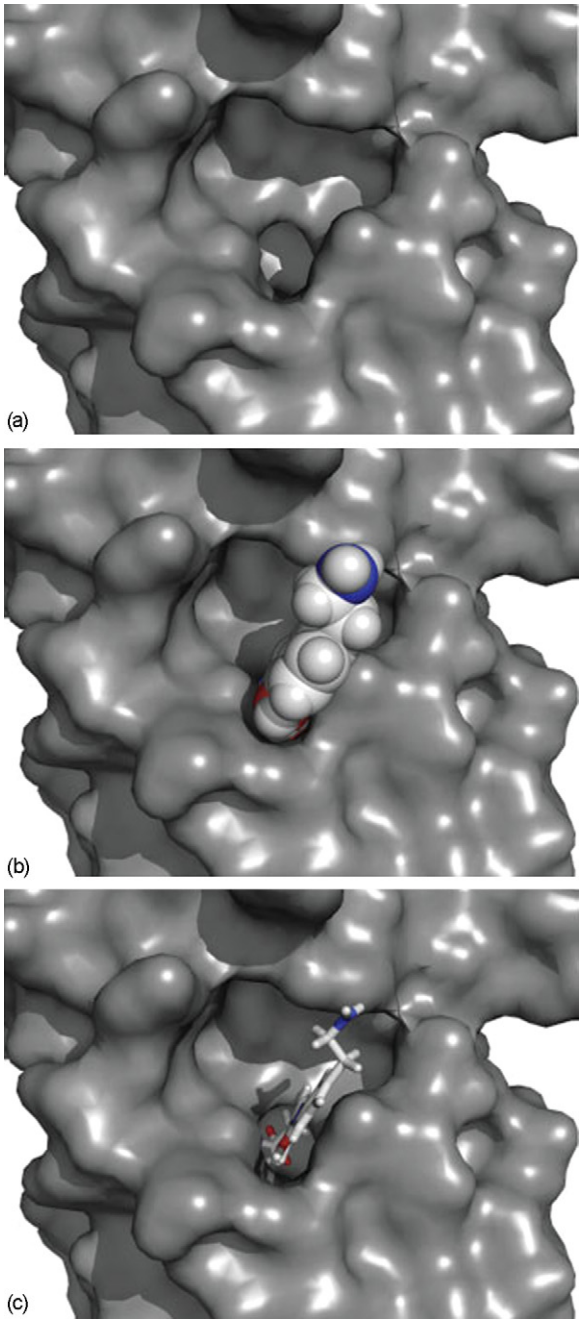

Fig. 6. Surface contour images of the modeled anti-D-amino acid structure (a) without the ligand, (b) with the docked ligand in a spherical representation and (c) with the docked ligand in a stick representation [38].

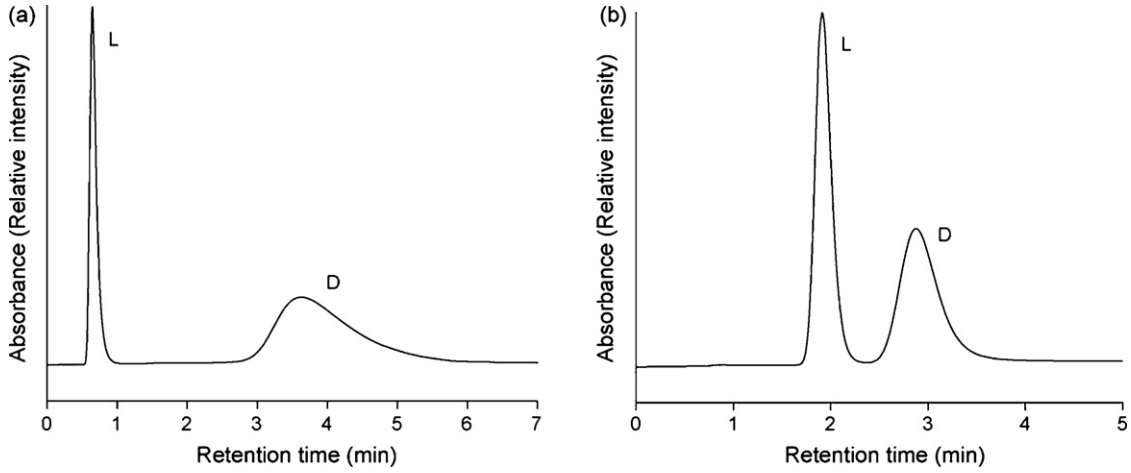

Fig. 7. Enantioseparations of (a) 4-hydroxymandelic acid at 3 mL/min and (b) vanillomandelic acid at 1 mL/min using phosphate-buffered saline as a mobile phase [41].

increase of the nonspecific interaction whereas, on the other hand, hindering the access to the specific catalytic site.

Furthermore, chiral recognition mechanism on PGA was investigated using molecular modeling and docking studies [29–31]. As depicted in Fig. 4a, the associated binding mode for (*S*)-2-(4-chlorophenyl)-2-phenoxyacetic acid is characterized by the presence of numerous H-bond interactions involving the carboxy group with both the backbone NH and the OH group of SerB386 [31]. An additional H-bond is established between the ether oxygen of the ligand and the ArgB263 side chain. Moreover, an ionic interaction is formed between the negatively charged carboxy group and the positively charged N-terminal SerB1. A set of charge-transfer interactions is also found between the ligand phenoxy and phenyl moieties and the PheB24, PheA146, PheB71 and PheB256 aromatic rings. Regarding (*R*)-2-(4-chlorophenyl)-2-phenoxyacetic acid, the calculated posing is similar to the one found for the (*S*)-isomer, even if the H-bond interaction between the ether oxygen and ArgB263 side chain is absent and the phenoxy moiety is placed in the same position occupied by the phenyl ring in the (*S*)-enantiomer (Fig. 4b) [31].

2.6. Antibodies

In 1982, Mertens et al. [32] raised polyclonal antibodies for (+)- and (−)-abscisic acid (ABA) in rabbit sera and prepared their immunoglobulin G (IgG) fractions. Then, the IgG antibodies bound to cyanogen bromide-activated Sepharose 4B were utilized for the isolation of ^{3}H-(+)-ABA as well as ^{3}H-(−)-ABA [32]. The antibody column could be reused at least 6–8 times without loss of capacity. Similarly, an antibody for (+)-ABA was produced using ABA-4′-*p*-aminobenzoyl-hydrazone coupled to keyhole limpet hemocyanin (KLH) as an immunogene [33]. The obtained monoclonal antibodies were bound to cyanogen bromide-activated Sepharose 4B. The target enantiomer was retained by the antibody CSPs and eluted second, while the opposite enantiomer eluted with the void volume. However, the above column was also used 10 times without loss of capacity. The major disadvantage of an antibody column is that for elution of a more retained enantiomer strong mobile phase is required, and that it is unstable for repeated uses.

Recently, Hofstetter et al. raised stereospecific antibodies against a broad class of substances, α-amino acids and applied to enatioseparations of α-amino acids [6,34–38]. *p*-Amino-D- and L-phenylalanine, respectively, were coupled to KLH or bovine serum albumin (BSA) via the *p*-amino group by diazotization of its tyrosyl residues, and the resulting conjugates, *p*-azo-D-phenylalanine-KLH or -BSA and *p*-azo-L-phenylalanine-KLH or -BSA, were used as the immunogenes for rabbits. The produced monoclonal antibodies were bound to Sepharose 4B [6], POROS-OH (poly(styrene-divinylbenzene) porous perfusion beads) [34,35] and silica gels [36–38] activated by DSC. Routine enantioseparations of α-amino acids could be achieved on antibodies bound to the latter two supports under true high-performance chromatographic conditions. Fig. 5 shows enantioseparations of tyrosine, phenylalanine and *p*-aminophenylalanine on a monoclonal antibody column against L-amino acid [34]. The obtained column gave the enantioselectivity for a wide variety of α-amino acids; the higher one for aromatic and bulky side-chain amino acids, and lower one for aliphatic amino acids [34]. Using an anti-D-amino acid antibody as a chiral selector, the L-enantiomers eluted with the void volume, while the D-enantiomers eluted second. Inverted elution orders were obtained on CSPs prepared from an anti-L-amino acid antibody [34]. The effect of the mobile phase parameters such as flow rate, temperature and organic modifier on the enantioseparations of various aromatic amino acids was examined [35]. The retention factor of the second eluted enantiomer is dependent on the affinity between the analyte and the immobilized antibody, while that was independent of the flow rate. With an increase of a column temperature, the retention factor of a solute decreased. However, van't Hoff plot was not linear. This means that at an elevated temperature, conformational changes of the immobilized antibody could occur [35]. Furthermore, addition of organic modifier did not improve the separation. The enantioselective binding of D-amino acids on the anti-D-amino acid antibody was investigated using molecular modeling and ligand docking [38]. The results indicates that in addition to four hydrogen bonds, formed between amino acid residues in the binding site and the ligand, a number of hydrophobic interactions are involved in the formation of the antibody-ligand complex as shown in Fig. 6 [38]. The aromatic side chain of the ligand interacts with Trp and Tyr residues in the binding site through π–π stacking.

Hofstetter and co-workers prepared CSPs based anti-α-hydroxy acids [39–41]. *p*-Amino-D- and L-phenyacetic acid, respectively, were coupled to KLH or BSA via the *p*-amino group by diazotization of its tyrosyl residues and were immunized to rabbits, as described for *p*-amino-phenylalanine [6]. The prepared monoclonal antibodies against D-α-hydroxy acids were bound to silica gels [39,40] or POROS-OH [41] activated by DSC. Chiral separations of several aliphatic and aromatic members of this class of compounds were achieved under mild isocratic buffer conditions using phosphate-buffered saline, pH 7.4, as the mobile phase.

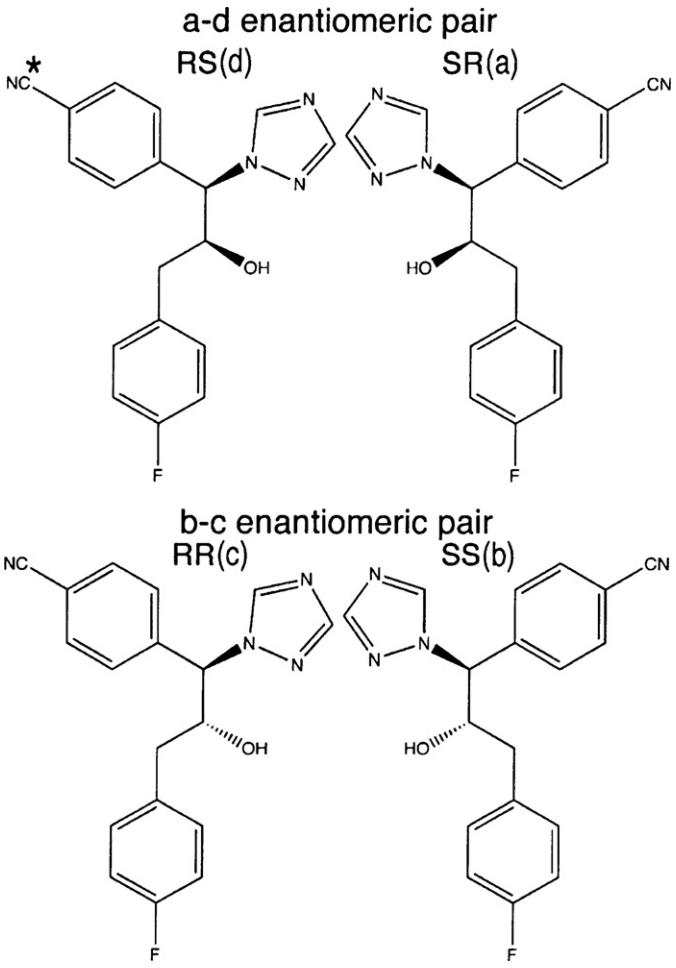

Fig. 8. The four stereoisomers (a–d) of the hapten, 4-[3-(4-fluorophenyl)-2-hydroxy-1-(1,2,4-triazol-1-yl)-propyl]benzonitrile (finrozole) [44]. The position of the linker, which was used to conjugate a protein for the development of the original monoclonal antibodies through immunization, is marked by an asterisk in the D-enantiomer form.

Fig. 7 shows enantioseparations of D,L-4-hydroxymandelic acid and D,L-vanillomandelic acid on a monoclonal antibody column to D-hydroxy acid [34]. No significant changes of column performance were not observed after 300 injections [41].

Recombinant antibody fragments (antigen binding fragment, Fab) have been prepared for finrozole (Fig. 8), which has two chiral centers, using genetic engineering techniques [42]. The obtained Fab, which has a molecular weight of about 30 kDa, was bound to Chelating Sepharose Fast Flow loaded with copper ions. Furthermore, single mutant (Tyr96Val) or double mutants (Tyr96Val/Trp33Ala mutants) of the Fab was prepared to increase the lifetime of the antibody fragment columns [43]. Decreasing the affinity by one or more orders of magnitude resulted in enabling the use of a lower concentration of organic solvent for elution. The crystal structure of this enantioselective Fab is determined in the absence or the presence of the hapten [44]. The hapten molecule was tightly bound in a deep cleft between the light and heavy chains of the Fab. The complex structure also allowed us to describe the molecular basis for enantioselectivity and to deduce the absolute configurations of all the four different stereoisomers (a–d) of finrozole. The antibody fragment selectively bound the SR (a) enantiomer from the racemic mixture of a and d-enantiomers. Asp95 and Asn35 of the H-chain in the antibody seem to provide this specificity through hydrogen bonding interactions [44].

Anti-D-methamphetamine monoclonal antibodies were immobilized onto Sepharose 4B activated by cyanogenbromide or amino-silica gels modified with glutaraldehyde [45]. The antibody-based CSPs were applied to enantioseparations of amphetamine and methamphetamine. Anti-3,3′,5-triiodo-L-thyronine (anti-L-T_3) polyclonal antibodies were immobilized onto amino-silica gels modified with glutaraldehyde [46]. The enantiomers of T_3 were baseline separated under mild continuous isocratic elution conditions using plain phosphate buffer, pH 7.4.

2.7. Other proteins

FABPs belong to a family of low molecular mass proteins, exhibiting high affinity binding constants for small endogenous and exogenous lipophilic ligands [7]. CSPs based on FABP from chicken liver were successfully applied to enantioseparations of some aryl- and aryloxypropionic acids (suprofen, flurbiprofen, 2-(3-ethylphenoxy)propionic acid, 2-(3-phenylphenoxy)propionic acid, 2-(3-phenoxyphenoxy)propionic acid and 2-(2,6-dimethylphenoxy)propionic acid) [7].

Avidin and streptavidin have very similar primary structure and are tetrameric proteins, being isolated from hen egg white and from the bacterium *Streptomyces avidinii*, respectively. Their isoelectric points are 10 and 5, respectively. The streptavidin-based CSPs were used for the separation of adenosine enantiomers [8]. On a streptavidin–biotin complex CSPs, it was demonstrated that the blockage of the biotin sites of the immobilized streptavidin was responsible for a strong decrease in the enantioselectivity via a direct and/or an indirect effect.

3. Conclusions

After 2001 serum albumin from other species, penicillin G-acylase, antibodies, fatty acid binding protein and streptavidin were newly introduced as the chiral selectors in CSPs. It is interesting that antibodies against α-amino acids and -hydroxy acids were produced and that the antibody-based CSPs could be utilized for enantioseparations of a member of these class of compounds. Furthermore, chiral recognition abilities of OMCHI-based CSPs, which are commercially available, come from cAGP, one of a lipocaline family.

The chiral recognition sites of some proteins were located and their chiral recognition mechanisms were elucidated by molecular modeling and ligand docking, and X-ray crystallography. However, further studies could be required for elucidating the chiral recognition mechanism of proteins or glycoproteins.

Recently, a protein or glycoprotein is utilized as a chiral selector in CE or chip. This trend will not change even in the future. However, protein-based CSPs will find their rooms in enantioseparations of some compounds.

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References

[1] J. Haginaka, J. Chromatogr. A 906 (2001) 253.
[2] M.C. Millot, J. Chromatogr. B 797 (2003) 131.
[3] G. Gübitz, M.G. Schmid (Eds.), Chiral Separations. Methods and Protocols, Humana Press, Totowa, 2004.
[4] I. Fitos, J. Visy, M. Simonyi, J. Biochem. Biophys. Methods 54 (2002) 71.
[5] G. Massolini, E. Calleri, E. De Lorenzi, M. Pregnolato, M. Terreni, G. Félix, C. Gandini, J. Chromatogr. A 921 (2001) 147.

[6] O. Hofstetter, H. Hofstetter, M. Wilchek, V. Schurig, B.S. Green, Int. J. Biochromatogr. 5 (2000) 165.
[7] G. Massolini, E. De Lorenzi, E. Calleri, C. Bertucci, H.L. Monaco, M. Perduca, G. Caccialanza, I.W. Wainer, J. Chromatogr. B 751 (2001) 117.
[8] C. Ravelet, M. Mchaud, A. Ravel, C. Grosset, A. Villet, E. Peyrin, J. Chromatogr. A 1036 (2004) 155.
[9] H. Hofstetter, O. Hofstetter, Trends Anal. Chem. 24 (2005) 869.
[10] R. Mallik, T. Jiang, D.S. Hage, Anal. Chem. 76 (2004) 7013.
[11] R. Mallik, D.S. Hage, J. Pharmaceut. Biomed. Anal. 46 (2008) 820.
[12] R. Mallik, C. Wa, D.S. Hage, Anal. Chem. 79 (2007) 1411.
[13] X.M. He, D.C. Carter, Nature 358 (1992) 209.
[14] I. Petitpas, A.B. Bhattacharya, S. Twine, M. East, S. Curry, J. Biol. Chem. 276 (2001) 22804.
[15] V.T.G. Chuang, M. Otagiri, Chirality 18 (2006) 159.
[16] H. Xuan, D.S. Hage, Anal. Biochem. 346 (2005) 300.
[17] R. Mallik, H. Xuan, D.S. Hage, J. Chromatogr. A 1149 (2007) 294.
[18] E. Hazai, J. Visy, I. Fitos, Z. Bikádi, M. Simonyi, Bioorg. Med. Chem. 14 (2006) 1959.
[19] F. Zsila, Y. Iwao, Biochem. Biophys. Acta 1770 (2007) 797.
[20] Y. Sadakane, H. Matsunaga, K. Nakagomi, Y. Hatanaka, J. Haginaka, Biochem. Biophys. Res. Commun. 295 (2002) 587.
[21] H. Matsunaga, J. Haginaka, J. Chromatogr. A 1106 (2006) 124.
[22] F. Zsila, H. Matsunaga, Z. Bikádi, J. Haginaka, Biochem. Biophys. Acta 1760 (2006) 1248.
[23] B. Stoffer, T.P. Frandsen, P.K. Busk, P. Schneider, I. Svendsen, B. Svensson, Biochem. J. 292 (1993) 197.
[24] A. Strandberg, A. Nyström, S. Behr, A. Karlsson, Chromatographia 50 (1999) 215.
[25] A. Karlsson, I. Arfwidsson, Z. Husovic, B. Svensson, Chromatographia 54 (2001) 717.
[26] G. Götmar, J. Samuelsson, A. Karlsson, T. Fornstedt, J. Chromatogr. A 1156 (2007) 3.
[27] J.L. Barbero, J.M. Buesa, G.G. de Buitrago, E. Méndez, A. Pérez-Aranda, J.L. Carcía, Gene 49 (1986) 69.
[28] E. Calleri, G. Massolini, D. Lubda, C. Temporini, F. Loiodice, C. Caccialanza, J. Chromatogr. A 1031 (2004) 93.
[29] G. Massolini, G. Fracchiolla, E. Calleri, G. Carbonara, C. Temporini, A. Lavecchia, S. Cosconati, E. Novellino, F. Loiodice, Chirality 18 (2006) 633.
[30] A. Lavecchia, S. Cosconati, E. Novellino, E. Calleri, C. Temporini, G. Massolini, G. Carbonara, G. Fracchiolla, F. Loiodice, J. Mol. Graph. Mod. 25 (2007) 773.
[31] C. Temporini, E. Calleri, G. Fracchiolla, G. Carbonara, F. Loiodice, A. Lavecchia, P. Tortorella, G. Brusotti, G. Massolini, J. Pharmaceut. Biomed. Anal. 45 (2007) 211.
[32] R. Mertens, M. Stuning, E.W. Weiler, Naturwissenschaften 69 (1982) 595.
[33] S.A. Quarrie, G. Galfre, Anal. Biochem. 151 (1985) 389.
[34] O. Hofstetter, H. Lindstrom, H. Hofstetter, Anal. Chem. 74 (2002) 2119.
[35] O. Hofstetter, H. Lindstrom, H. Hofstetter, J. Chromatogr. A 1049 (2004) 85.
[36] J.M. Zeleke, G.B. Smith, H. Hofstetter, O. Hofstetter, Chirality 18 (2006) 544.
[37] E.J. Franco, H. Hofstetter, O. Hofstetter, J. Sep. Sci. 29 (2006) 1458.
[38] D.I. Ranieri, D.M. Corgliano, E.J. Franco, H. Hofstetter, O. Hofstetter, Chirality 20 (2008) 559.
[39] T.K. Zeleke, J.M. Zeleke, H. Hofstetter, O. Hofstetter, J. Mol. Recog. 18 (2005) 334.
[40] H. Hofstetter, J.R. Cary, P.P. Eleniste, J.R. Hertweck, H.J. Lindstrom, D.I. Ranieri, G.B. Smith, L.P. Undesser, J.M. Zeleke, T.K. Zeleke, O. Hofstetter, Chirality 17 (2005) S9.
[41] E.J. Franco, H. Hofstetter, O. Hofstetter, J. Pharmaceut. Biomed. Anal. 46 (2008) 907.
[42] T.K. Nevanen, L. Söderholm, K. Kukkonen, T. Suortti, T. Teerinen, M. Linder, H. Söderlund, T.T. Teeri, J. Chromatogr. A 925 (2001) 89.
[43] T.K. Nevanen, M.-L. Hellman, N. Munck, G. Wohlfahrt, A. Koivula, H. Söderlund, Protein Eng. 16 (2001) 1089.
[44] T. Parkkinen, T.K. Nevanen, A. Koivula, J. Rouvinen, J. Mol. Biol. 357 (2006) 471.
[45] A.C. Lua, T.-Y. Chou, J. Chromatogr. A 967 (2002) 191.
[46] J. Koidl, H. Hödl, M.G. Schmid, M. Konrad, S. Petschauer, G.M. Kostner, G. Gübitz, J. Biochem. Biophys. Methods 69 (2006) 33.