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Journal of Chromatography A, 906 (2001) 253–273

JOURNAL OF CHROMATOGRAPHY A

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Review

Protein-based chiral stationary phases for high-performance liquid chromatography enantioseparations

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Abstract

The enantioseparations of various compounds using proteins as the chiral selectors in high-performance liquid chromatography (HPLC) are considered in this review. The proteins used include albumins such as bovine serum albumin and human serum albumin, glycoproteins such as α_1-acid glycoprotein, ovomucoid, ovoglycoprotein, avidin and riboflavin binding protein, enzymes such as trypsin, α-chymotrypsin, cellobiohydrolase I, lysozyme, pepsin and amyloglucosidase, and other proteins such as ovotransferrin and β-lactoglobulin. This review deals with the properties of HPLC chiral stationary phases based on proteins, and the enantioselective properties and chiral recognition mechanisms of these stationary phases.

Keywords: Reviews; Chiral stationary phases, LC; Enantiomer separation; Proteins; Glycoproteins; Enzymes

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)00504-5

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1. Introduction

A protein and glycoprotein, respectively, consists of amino acids, and amino acids and sugars, both of which are chiral. Thus, all proteins have the ability to discriminate a chiral molecule. However, only a limited number of proteins have been investigated as HPLC chiral stationary phases (CSPs). Protein-based CSPs are of special interest because of their unique enantioselective properties and because they are suitable for separating a wide range of enantiomeric forms [1,2]. Those developed so far have included albumins such as bovine serum albumin (BSA) [3] and human serum albumin (HSA) [4], glycoproteins such as α_1-acid glycoprotein (AGP) [5], ovomucoid from chicken egg whites (OMCHI) [6], ovoglycoprotein from chicken egg whites (OGCHI) [7], avidin (AVI) [8] and riboflavin binding protein (RfBP) (or flavoprotein) [9], enzymes such as trypsin [10], α-chymotrypsin [11], cellobiohydrolase I (CBH I) [12], lysozyme [13], pepsin [14], and amyloglucosidase [15] and other proteins such as ovotransferrin (or conalbumin) [16] and β-lactoglobulin [17]. The physical properties of these proteins are shown in Table 1. CSPs based on BSA, HSA, AGP, OMCHI, AVI, CBH I and pepsin are now commercially available as shown in Table 2.

The advantages of protein-based CSPs generally include the use of an aqueous mobile phase, as for reversed-phase HPLC, enantioselectivity for a wide range of compounds and direct analysis without derivatization. The disadvantages have included low capacity, lack of column ruggedness and limited understanding of the chiral recognition mechanism. Thus, the protein-based CSPs are useful for analytical purposes, but are not generally applicable to preparative isolation. To stabilize the protein-based CSPs, crosslinking of the protein by glutaraldehyde has been tried [18]. Further, CSPs based on a protein fragment or protein domain have been prepared [19–21]. These might be of higher capacity because only the active protein mass is used. Also, it is possible to

Table 1
Physical properties of proteins used for HPLC chiral stationary phases

Protein	Molecular mass	Carbohydrate content (%)	Isoelectric point	Origin
Albumins				
BSA	66 000		4.7	Bovine serum
HSA	65 000		4.7	Human serum
Glycoproteins				
α_1-Acid glycoprotein (AGP)	41 000	45	2.7	Human or bovine serum
Ovomucoid (OMCHI)	28 000	30	4.1	Egg white
Ovoglycoprotein (OGCHI)	30 000	25	4.1	Egg white
Avidin (AVI)	66 000	7	10.0	Egg white
Riboflavin binding protein (flavoprotein)(RfBP)	32 000–36 000	14	4	Egg white
Enzymes				
Cellobiohydrolase I (CBH I)	64 000	6	3.9	Fungus
Lysozyme	14 300		10.5	Egg white
Pepsin	34 600		<1	Porcine stomach
Amyloglucosidase	97 000	10–35	4.2	Fungus
Others				
Ovotransferrin (conalbumin)	77 000	2.6	6.1	Egg white
β-Lactoglobulin	18 000/36 000		5.2	Bovine milk

Table 2
Commercially available protein-based HPLC chiral stationary phases

Protein	Trade name	Manufacturer
BSA	RESOLVOSIL BSA-7, BSA-7PX	Nagel
	ULTRON ES-BSA	Shinwa Chemical Industries
	CHIRAL BSA	Shandon
HSA	CHIRAL HSA	Shandon
	CHIRAL-HSA	ChromTech AB
AGP	CHIRAL-AGP	ChromTech AB
OMCHI	ULTRON ES-OVM	Shinwa Chemical Industries
AVI	Bioptic AV-1	GL Sciences
CBH I	CHIRAL-CBH	ChromTech AB
Pepsin	ULTRON ES-PEPSIN	Shinwa Chemical Industries

understand the chiral recognition site(s) of protein-based CSPs by investigating whether or not independent chiral binding sites exist on each fragment or domain [21].

Previously, many book chapters and review articles have dealt with CSPs based on proteins [1,2,22–27]. This review article deals with the properties of HPLC CSPs based on proteins, and the enantioselective properties and chiral recognition mechanisms of these CSPs.

2. Preparation of chiral stationary phases based on a protein

It is well known that many factors such as the physical properties of the base materials, spacer length and binding method affect the resolution of enantiomeric forms on protein-based CSPs [28–30]. There are two methods for the preparation of protein-based CSPs: in one the protein is physically adsorbed onto the base materials, and in the other the protein is covalently bound to the base materials. Agarose, silica gels and polymers have been used as the base materials for the immobilization of a protein [31]. Among these, silica gels have mainly been used for the base materials. The disadvantage of silica-based stationary phases is that the eluent pH is limited to the range 2 to 8. However, in strong acidic or alkaline solution, a protein sometimes undergoes denaturation. The separation of enantiomers on a protein-based CSP is generally attained using an eluent whose pH is between 3 and 8. Thus, the optimal eluent pH for the enantioseparation of a solute on a protein-based CSP could be within the pH ranges used for silica-based materials.

With regard to the physical adsorption of a protein on the base materials, underivatized silica gels have been used as the base materials [32]. The disadvantages of the adsorption method are that multilayer adsorption of the protein on the base materials may occur, and that the adsorbed protein can be eluted. It is better to bind a protein covalently to the base materials.

Proteins are covalently bound to derivatized silica gels via the amino or carboxyl group of the protein. Fig. 1A and B show the typical preparation method for protein-based CSPs via an amino group of a protein. In Fig. 1A, the method includes activation of porous aminopropyl-silica gels by *N*,*N*′-disuccinimidylcarbonate (DSC), binding of the protein and blocking of the activated amino groups [7]. In this case, a side-chain amino group(s) of a protein such as Lys and Arg, and/or an N-terminal amino group, could be used for binding of the protein to the activated silica gel. Instead of DSC, *N*,*N*′-disuccinimidylsuberate (DSS) was used for activation of aminopropyl-silica gels [33]. The protein was bound to DSC-activated aminopropyl-silica gels via a urea bond, while the six CH_2 chains remained between two amide bonds in the case of DSS. This long hydrophobic spacer may take part in hydrophobic interactions with the solute, resulting in lower enantioselectivity of the solute than a less hydrophobic spacer [33]. Otherwise, higher enantioselectivity could be obtained because of the increased flexibility and availability of the ligand [34]. Glycerylpropyl-silica (diol-silica) gels activated with 1,1′-carbonyl-

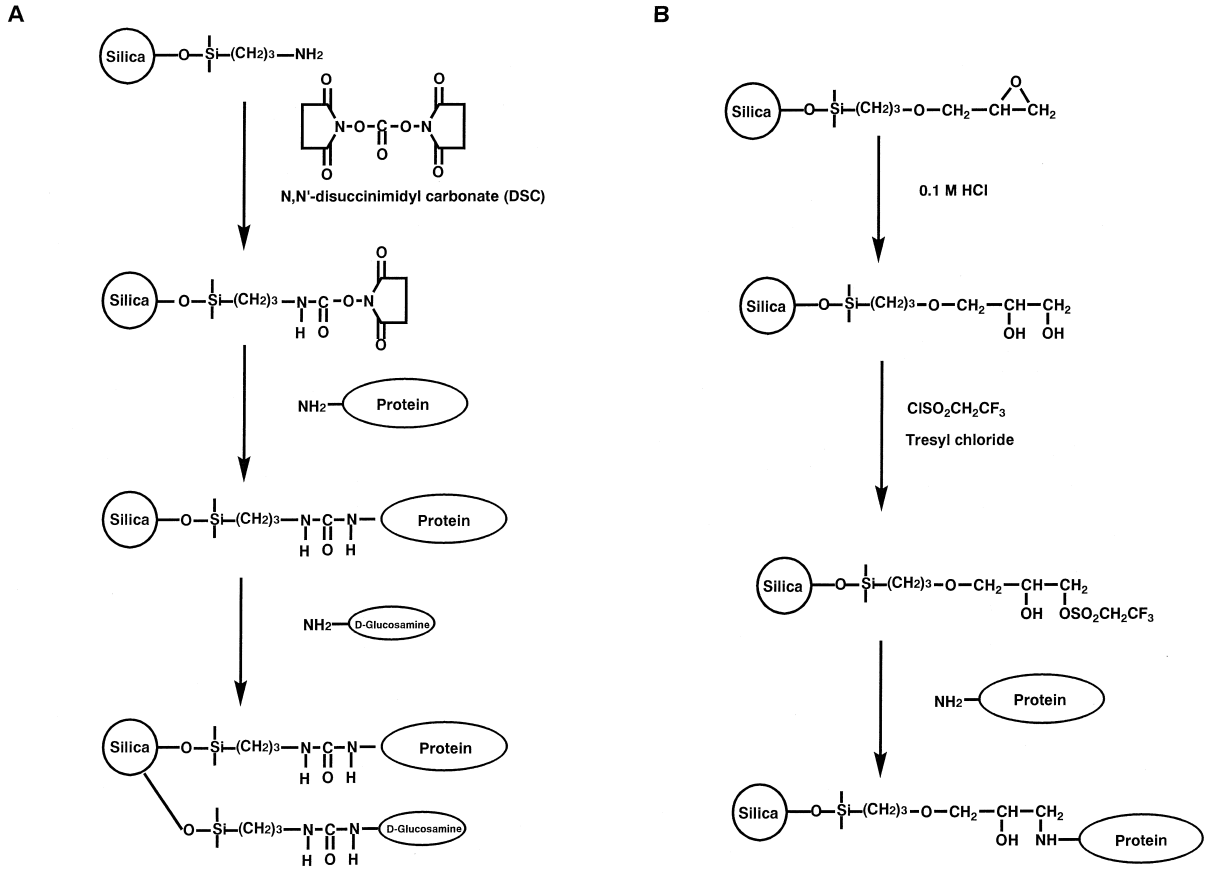

Fig. 1. Synthesis scheme for the preparation of protein-based chiral stationary phases. (A,B,C) Via the amino group of the protein; (D) via the carboxyl group of the protein.

diimidazole were used for the preparation of protein-based CSPs, where the protein was bound to base materials via the amino or carboxyl group(s) of the protein [4].

Another preparation method for protein-based CSPs via an amino group of a protein is shown in Fig. 1B. Glycidoxypropyl-silica (epoxy-silica) gels were hydrolyzed with hydrochloric acid. The obtained glycerylpropyl-silica gels were activated with tresyl chloride, and reacted with a protein, where the protein was bound to the base materials via an amine bond [29]. It is possible to react glycidoxypropyl-silica gels directly with the protein without tresyl chloride activation [29]. Furthermore, glyceryl-propyl-silica gels were oxidized to their aldehyde forms by periodic acid. The protein was immobilized to the aldehyde-silica gels followed by reductive amination in the presence of sodium cyanoborohydride [29]. In addition, proteins can be bound to aminopropyl-silica gels using glutaraldehyde as crosslinker, resulting in crosslinking by Schiff-base formation, as shown in Fig. 1C. The obtained imino functions are further reduced using sodium cyanoborohydride [1].

On the other hand, using water-soluble carbodiimide and *N*-hydroxysulfosuccinimide (HSSI) the carboxyl group of the protein can be bound to aminopropyl-silica gels, as shown in Fig. 1D [20]. In this case, the protein is immobilized to the base materials via an amide bond.

The chiral recognition properties of the adsorbed or bound protein may be different from those of the protein in solution because of blocking of functional groups and/or conformational changes. The bound

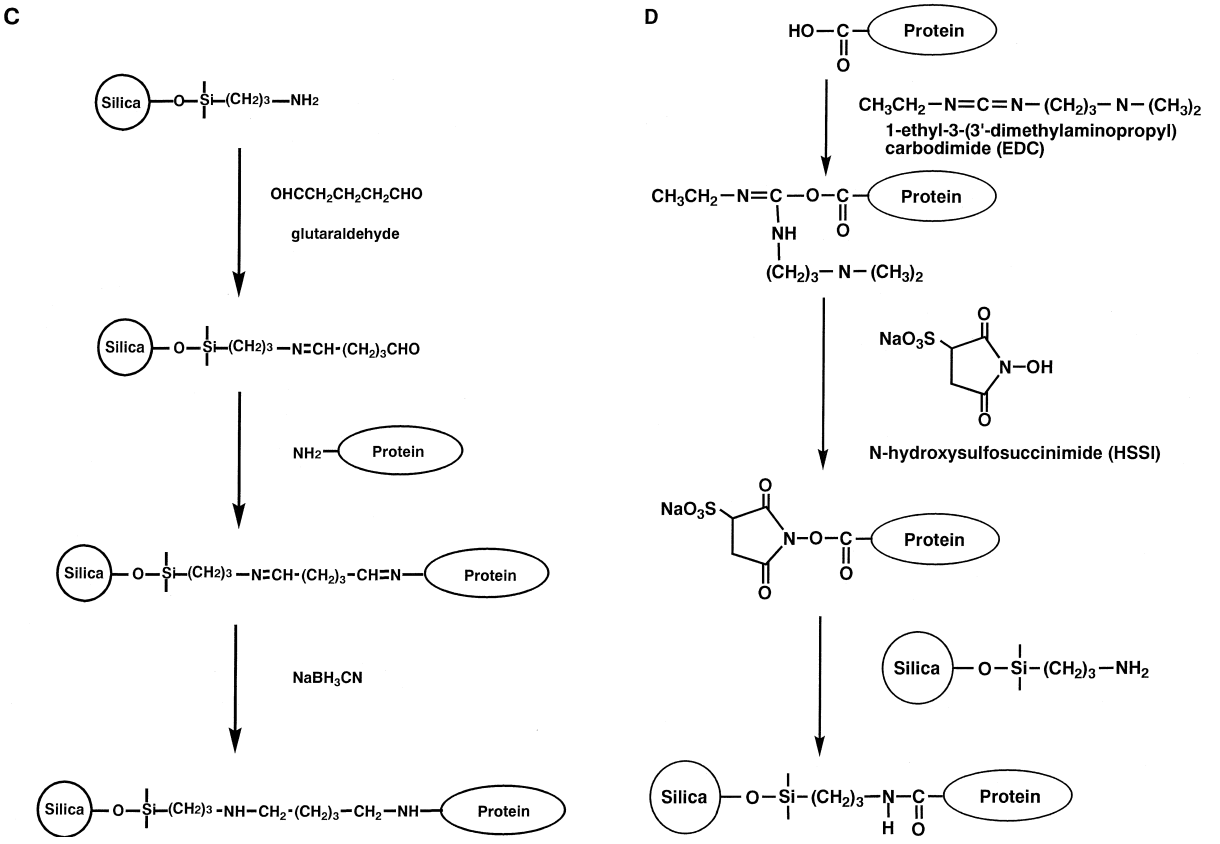

Fig. 1. (*continued*).

protein is often more stable against changes in eluent pH and composition compared to the protein in solution.

3. Types of protein-based chiral stationary phases

3.1. Chiral stationary phases based on albumins

3.1.1. Bovine serum albumin

In 1973, BSA-agarose was used for the enantioseparation of tryptophan, which is the first report of the use of a protein-based CSP for chiral resolution purposes [35]. D- and L-tryptophan were clearly resolved using this CSP, and the D-form was eluted first, consistent with previous binding studies in solution [22]. In the following years, HPLC CSPs based on BSA were developed and used for the separation of a variety of enantiomers, which included N-derivatized amino acids, aromatic amino acids, uncharged solutes, sulfoxides and sulfoximine derivatives [1,2,36]. Only a few of the racemic amines investigated included prilocain, where the eluent pH used is 8.9 [36]. There are essentially three variables by means of which the mobile-phase system can be optimized for a particular resolution problem, viz. eluent pH, ionic strength and organic modifier [37]. An optimization procedure in chiral resolution using a systematic change of the mobile-phase composition was proposed for a BSA column [1].

Agarose, silica gels and polymers have been used as the base materials for the immobilization of BSA [31]. The polymers used include hydroxyethylmethacrylate [38], polystyrene–divinylbenzene perfusion beads [39] and polyethylene hollow-fiber membranes [31,40]. Nakamura et al. [31,40] pre-

pared a BSA-multilayer-adsorbed porous hollow-fiber membrane as a CSP. Fig. 2 shows the chiral resolution of tryptophan on a BSA-monolayered- and BSA-four-layered-adsorbed porous hollow-fiber module. The former exhibited an enantioseparation factor of 2.9, while the latter exhibited a higher enantioseparation factor of 6.6. BSA adsorbed in the multilayers by the polymer chains grafted onto the pore surface resulted in a prolongation of the retention time of L-tryptophan. The BSA-multilayered porous hollow-fiber membrane gave a satisfactory resolution for tryptophan.

Erlandsson and Nilsson [19] and Andersson et al. [41] isolated a BSA fragment of molecular mass ca. 38 000 by enzymatic cleavage, and crosslinked the fragment into aminopropyl-silica materials by glutaraldehyde or adsorbed it on silica materials.

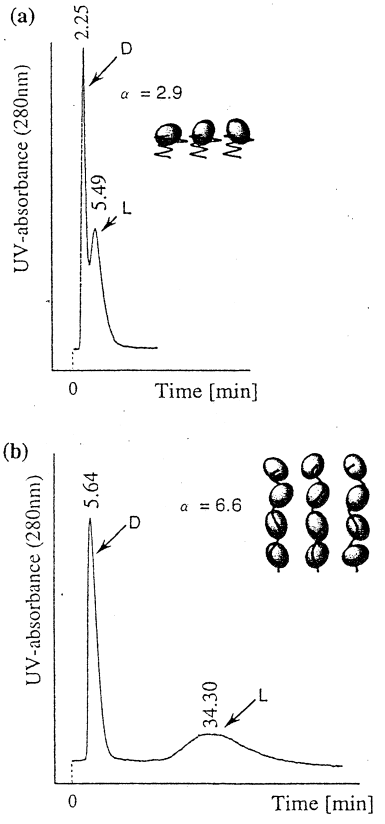

Fig. 2. Enantioseparation of tryptophan during the permeation of a racemic solution through a BSA-multilayered porous hollow-fiber membrane. Reproduced, with permission, from Ref. [40]. Degree of multilayer binding: (a) monolayer; (b) four layers.

However, the BSA-fragment column obtained had less capacity and enantioselectivity, and was less stable than the intact BSA column. Haginaka and Kanasugi [42,43] isolated a BSA fragment of molecular mass 35 236 estimated by electrospray ionization mass spectrometry. The estimated molecular mass was in good agreement with the predicted molecular mass (35 234) of the half-cystinyl BSA fragment, which is ascribable to amino acid sequences 1–307 having eight disulfide bonds and one half-cystinyl bond in the 34th residue. Previously, the amino acid sequence of BSA was determined to consist of 582 amino acid residues [44]. Recently, Hirayama et al. [45] reported that the 156th tyrosine residue in was lacking in the previous sequence, and that BSA consists of 583 amino acid sequences. Thus, the cleavage was between 307 and 308 (Asp and Phe), which corresponds to 306 and 307 in the previous sequence. Next, the intact and fragmented BSA were bound to DSC-activated aminopropyl-silica gels. The bound amounts of the BSA fragment were 2.2–2.7 times more than that of the intact BSA. Chiral resolution of 2-arylpropionic acid derivatives, benzodiazepines, warfarin and benzoin was attained with the BSA fragment columns. Fig. 3 shows chromatograms of benzoin using the BSA and BSA-fragment columns. The BSA-fragment columns gave higher enantioselectivity for lorazepam and benzoin because of the increased amounts bound, and lower enantioselectivity for the other compounds tested, compared with the BSA column. The lower enantioselectivity might be due to changes in the globular structure of the BSA fragment and/or changes in the local environment around the binding sites. Similar results were obtained with the enantioselective binding of HSA fragments. Soltes and Sebille [46] investigated the enantioselectivity of the reversible binding interaction between D- and L-tryptophan enantiomers and HSA fragments. All the HSA fragments had a weak yet non-zero enantioselectivity with regard to their preferential association with D- or L-tryptophan enantiomers.

3.1.2. Human serum albumin

CSPs based on HSA were first introduced by Domenici et al. [4]. A variety of weakly acidic and neutral compounds, which include 2-arylpropionic acid derivatives such as naproxen, flurbiprofen,

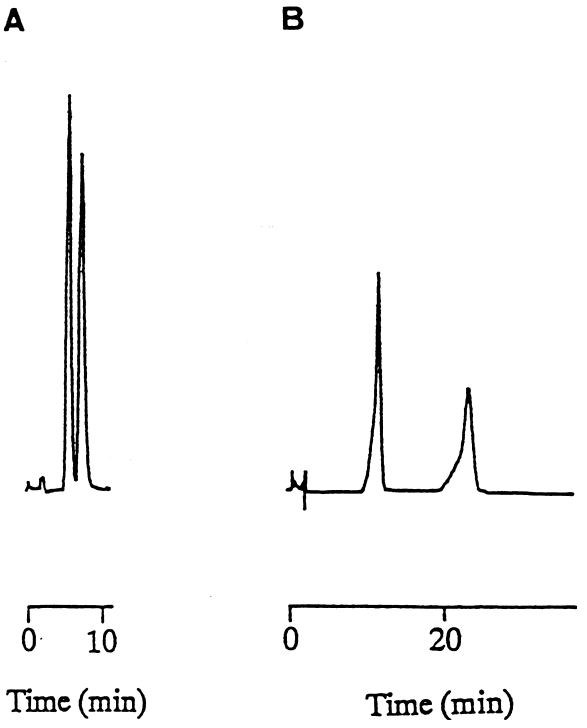

Fig. 3. Chromatograms of benzoin on the BSA (A) and BSA-fragment (B) columns. Reproduced, with permission, from Ref. [43]. HPLC conditions: column, 2.1 mm I.D.×100 mm; eluent, 50 m*M* phosphate buffer (pH 7.5)–1-propanol (96:4, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 254 nm; injected amount, 0.1 μg each.

ibuprofen, ketoprofen and fenoprofen, reduced folates such as leucovorin and 5-methyltetrahydrofolate, and benzodiazepines such as oxazepam, lorazepam and temazepam, are resolved on HSA-based CSPs [2]. HSA is closely related to BSA and, consequently, the stereoselective binding characteristics of these two proteins are similar. Sometimes, the elution order is reversed between CSPs based on these proteins; on HSA-based CSPs, (*S*)-warfarin elutes before (*R*)-warfarin, whereas on BSA-based CSPs the opposite elution order is observed [4]. These observations are consistent with the enantioselectivity of the native proteins [47].

The stereoselective binding characteristics of HSA have been thoroughly examined. The stereoselective binding of drugs on HSA occurs principally at two major binding sites: warfarin-azapropazone and indol-benzodiazepine sites (well known as sites I and II) [48]. In addition, other minor sites have been postulated to elucidate the binding of drugs whose binding could not be explained by the two major biding sites. CSPs based on HSA provide useful information on the affinity and binding mechanism of small molecules to HSA. Fig. 4 shows the enantioseparation of lorazepam hemisuccinate with and without addition of warfarin on a HSA column. The result indicates that the binding of warfarin caused an allosteric change in binding site II, leading to higher affinity of (*S*)-lorazepam hemisuccinate [49]. Furthermore, the binding of octanoic acid to HSA caused an allosteric change in binding site II, resulting in the displacement of suprofen and ketoprofen, and possibly (*S*)-oxazepam hemisuccinate and tolbutamide. At higher concentrations, the acid appeared to bind directly at site I, from which it is able to displace target solutes in a competitive fashion [50].

Recently, He and Carter determined the three-dimensional structure of HSA, which shows that binding sites I and II are located in hydrophobic cavities in subdomains IIA and IIIA, respectively [51]. The binding interaction of 2,3,5-triiodobenzoic acid in subdomain IIA includes hydrophobic interac-

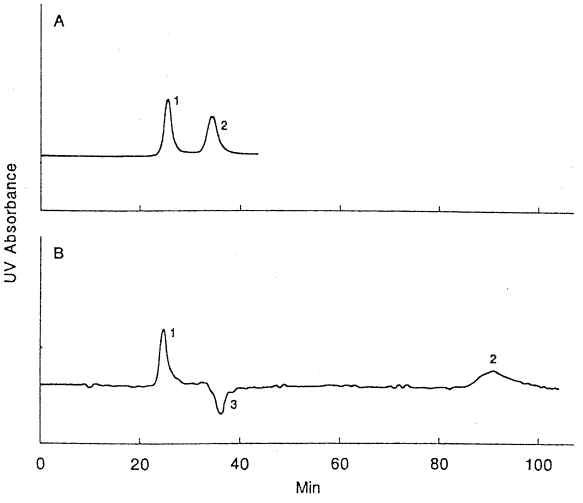

Fig. 4. Chromatograms of racemic lorazepam hemisuccinate on a HSA column without (*S*)-warfarin in the mobile phase (A) and with 40 μ*M* (*S*)-warfarin in the mobile phase (B). Reproduced, with permission, from Ref. [49]. Loaded amount: 2 μg. Key: 1=(*R*)-lorazepam hemisuccinate, 2=(*S*)-lorazepam hemisuccinate, 3=systemic peak of (*S*)-warfarin.

tions with residues Leu 219, Phe 223, Leu 234, Leu 238, Leu 260, Ala 261, Ile 264, Ile 290, Ala 291 and the hydrocarbon chain of Glu 292, and electrostatic or hydrogen bonding interactions with Arg 257, Arg 222 and Lys 199, and His 242. The chemistry of ligand binding in site II is analogous to that of site I. Fig. 5 shows a stereoview of HSA showing the various ligand-binding sites [52].

Yang and Hage indicated that both D- and L-tryptophan bind to single but distinct sites on HSA from frontal and zonal elution studies [53–55], and that (*R*)- and (*S*)-ibuprofen had one common binding site in addition to at least one other major binding region for (*S*)-ibuprofen [56]. Further, they characterized the kinetic and thermodynamic processes involved in the binding and separation of (*R*)- and (*S*)-warfarin on an HSA column [57,58]. They concluded that (*R*)- and (*S*)-warfarin bound to the same site of HSA, and that (*R*)- and (*S*)-warfarin interacted with regions on the interior and exterior of HSA, respectively. Peyrin et al. [59,60] described a binding mode of dansyl amino acids on the site II cavity where the hydrophobic groups of a compound occupied the non-polar interior of the cavity and carboxylate and sulfanilamide groups interacted with Arg 410 and Tyr 411 residues on the cavity rim forming electrostatic and hydrogen bonds.

The in situ derivatization of Cys 34 of HSA with ethacrylic acid was found to be a useful method to improve the retentivity and enantioselectivity of a solute on the HSA column [61]. The retention times were significantly shorter for most of the solutes tested, and the enantioselectivity was enhanced in many cases. Furthermore, the non-covalent binding of ethacrylic acid to HSA modified the binding properties. Ethacrylic acid, added to the eluent, interacted with the important binding sites, resulting in a marked increase of the enantioselectivity in the binding of (*R*)- and (*S*)-warfarin and a marked decrease of the enantioselectivity in the case of chiral benzodiazepines [61]. Further, it was found that HSA of which the Trp 214 residue is chemically derivatized with *o*-nitrophenylsulfenyl chloride, gave the same number of binding sites as intact HSA for (*R*)-warfarin and L-tryptophan (i.e., probes for the warfarin and indole sites, sites I and II, of HSA), but lower association equilibrium constants for both of these test solutes [62]. The results for (*R*)-warfarin are due to the direct blocking of HSA's warfarin binding region (site I) by the derivatized tryptophan residue, while the decrease in L-tryptophan retention is due to an allosteric-induced change in the indole binding region of HSA (site II).

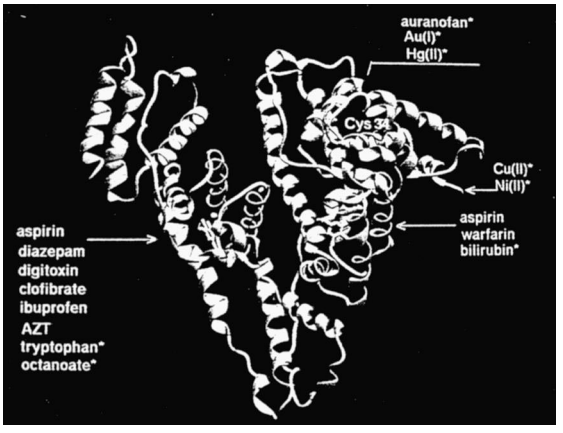

Fig. 5. Illustration summarizing the various ligand-binding sites on HSA. Reproduced, with permission, from Ref. [52]. Asterisks denote binding sites that can be inferred; all others have been determined crystallographically.

3.2. Chiral stationary phases based on glycoproteins

3.2.1. α_1-Acid glycoprotein

AGP is composed of a single peptide chain containing 181 amino acid units and five heteropolysaccharide units, which include 14 residues of sialic acid, giving the protein a very acidic character with an isoelectric point (p*I*) of 2.7. Since the molecular mass of AGP from human plasma ranges from 38 800 to 48 000, it is generally assumed to be 40 000 [63]. Recently, Haginaka and Matsunaga, using matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, reported that the average molecular mass of AGP could be ca. 33 000 and that the sugar content of AGP was estimated to be 34% [64]. AGP is the major plasma protein responsible for the protein binding of cationic drugs because AGP has a lower p*I* value than BSA and HSA, as shown in Table 1 [63].

CSPs based on AGP were developed by Hermansson [11]. Since that time there have been numerous

applications involving enantiomeric purity determinations of drugs and determination of drug enantiomers in biological fluids [65]. A wide range of basic, neutral and acidic compounds have been enantioseparated using AGP columns [2,22]. Various factors, such as eluent pH, type and concentration of organic modifier and charged modifier, ionic strength and temperature, affect the retentivity and enantioselectivity of solutes on AGP columns [65]. The hydrogen bonding properties and hydrophobicity of the uncharged organic modifier influence the enantioselectivity of the solute significantly [65]. Verapamil was not enantioseparated with a mobile phase containing 1-propanol, but was completely resolved with a mobile phase containing acetonitrile. Enantioseparation of methylphenobarbital with a pure phosphate buffer (pH 7.0) as eluent resulted in no resolution at all, while addition of only 2% 2-propanol to the mobile phase resulted in an induction of the enantioselectivity and complete resolution of the enantiomers (Fig. 6) [65]. Furthermore, addition of charged modifiers such as *N*,*N*-dimethyloctylamine, tetrapropylammonium bromide, tetrabutylammonium bromide and sparteine affected the retentivity and enantioselectivity of uncharged, anionic and cationic solutes [65–67]. These modifiers compete with the solute enantiomers at binding site(s) or bound to allosteric site(s). Further, the adsorption of these modifiers on the protein induces a reversible change in its conformation. With regard to a conformational change of AGP, it was reported that a reversible conformational change of immobilized AGP occurs between pH 5 and 7 [68]. Recently, a conformational change of immobilized AGP by temperature was reported by Waters et al. [69]. The enantioseparation of a model ligand was entropy-driven at lower temperature, while the enantioseparation at higher temperature was enthalpy-driven. The transition from the entropy- to the enthalpy-driven separation region suggested that the immobilized AGP underwent a conformational change.

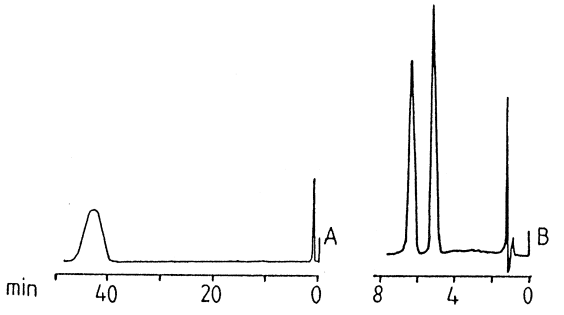

Fig. 6. Induction of enantioselectivity of methylphenobarbital by addition of 2-propanol to the mobile phase on an AGP column. Reproduced, with permission, from Ref. [65]. Mobile phase: A, 0.01 *M* phosphate buffer, pH 7.0; B, 2% 2-propanol in 0.01 *M* phosphate buffer, pH 7.0.

It was thought that drug binding to AGP occurred at a single hydrophobic pocket or cleft within the protein domain of the molecule [63]. Alternatively, more than one binding site was presented [63]. It is thought that the hydrophobic, electrostatic and hydrogen bonding interactions could play an important role in the retentivity and enantioselectivity of a solute on an AGP column [65]. There is no precise information on the chiral recognition sites and mechanism of AGP because of the lack of a tertiary structure. Further, the role of sugar moieties in enantioselective bindings by AGP has not been investigated precisely. Shiono et al. [70] reported that sialic acid residues influenced the enantioselective binding of basic drugs in different ways. They were not involved in enantioselective verapamil–AGP binding, and participated in the binding of (*S*)-propranolol but not of (*R*)-propranolol. Further, the role of the branching glycan structure of AGP in the interaction with propranolol and verapamil was investigated in terms of enantioselectivity in binding ability [71]. The results suggested that the branching type of glycan chains of AGP do not play a significant role in the chiral recognition in binding these basic drugs. Haginaka and Matsunaga [64] prepared partially deglycosylated (pd)-AGP by removal of a sugar moiety of AGP by treatment with *N*-glycosidase. The average molecular mass of pd-AGP was estimated to be ca. 30 600 by MALDI-TOF mass spectrometry. Fig. 7A and B show chromatograms of ethotoin enantiomers on AGP and pd-AGP columns, respectively. The retentivity and enantioselectivity of the neutral, acidic and basic solutes tested on the pd-AGP column were significantly or not significantly greater in most solutes than those on the native AGP column. This might be ascribable to the fact that, by cleavage of a sugar chain(s), pd-AGP may become more hydrophobic than AGP, and/or that a solute could easily be

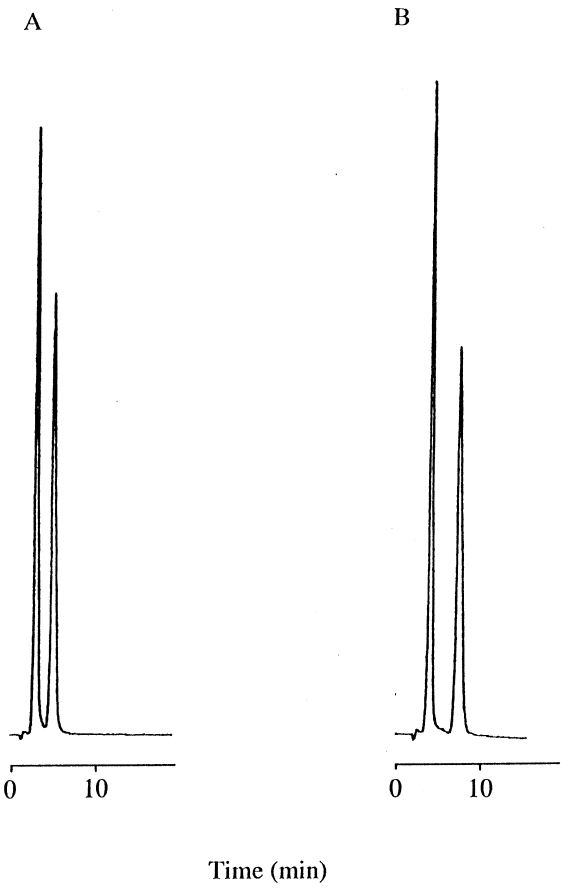

Fig. 7. Chromatograms of ethotoin enantiomers on AGP (A) and pd-AGP (B) columns. Reproduced, with permission, from Ref. [64]. HPLC conditions: column size, 2.0 mm I.D.×100 mm; eluent, 10 m*M* phosphate buffer (pH 5.0); flow-rate, 0.2 ml/min; detection, 210 nm; temperature, 25°C; loaded amount, 100 ng.

accessible to the specific and/or non-specific binding sites of pd-AGP [64]. Further study is required to clarify the chiral recognition mechanism of AGP and the role of the sugar moiety in chiral recognition.

3.2.2. Ovomucoid and ovoglycoprotein

CSPs based on OMCHI were developed by Miwa et al. [6]. The column was utilized for the resolution of acidic, basic and neutral enantiomers in bulk drugs and formulations [72,73], and the assay of enantiomers in biological fluids [74]. Kirkland et al. [75,76] reported that the OMCHI column provided better long-term stability for repetitive injections compared with the AGP column, and that column stability proved superior to other protein-based columns tested.

Various ovomucoids such as ovomucoid from turkey egg white (OMTKY) [21] and OMCHI [77] exist as three tandem, independent domains [78]. In order to gain information regarding the enantioselective recognition mechanism of ovomucoid proteins, it is of interest to know whether chiral recognition is expressed by all three domains, by only one domain, or by a combination of domains. Each domain and combination of domains, first and second, second and third domains, were isolated, purified and characterized [21]. Further, columns were made with purified OMTKY and OMCHI domains to test chiral recognition properties [21,71]. The third domain of OMTKY and OMCHI consisted of glycosylated (OMTKY3S and OMCHI3S) and unglycosylated domains (OMTKY3 and OMCHI3). The third domains of the OMTKY and OMCHI domains were found to be enantioselective to at least two classes of compounds, benzodiazepines and 2-arylpropionic acid derivatives. Glycosylation of the third domain did not affect chiral recognition. Further, the chiral recognition mechanism of OMTKY3 was elucidated using NMR measurements, molecular modeling and computational chemistry [21]. Fig. 8 illustrates the binding orientations of the enantiomers of U-80413, which is a 2-arylpropionic acid derivative, in each of the two surface regions identified on OMTKY3 [21]. The first group of amino acids, Val 6, Arg 21, Pro 22, Leu 23, Ly34 and Phe 53, is on the left, while the second group, Val 41, Val 42, Leu, 48 and Lue 50, is on the right. The tubular structure represents the protein backbone, and side chains of selected amino acid residues (Arg 21, Lys 34 and Phe 53) are shown. The peptide strand is wrapped around an α-helix and held in place by three disulfide bonds. The N-terminal is at the top right and the C-terminal is at the back. The dockings in the second site to the right were of higher energy and without apparent points of interaction that could produce chiral recognition. This site could account for the non-specific binding site. The selected, specific binding model for each of the (*R*)- and (*S*)-U-80413 with OMTKY3 is shown in Fig. 9 [21]. One can see similarities and differences in orientation and intermolecular interactions between (*R*)- and (*S*)-U-80413. The carboxyl groups of each enantiomer engage in electrostatic

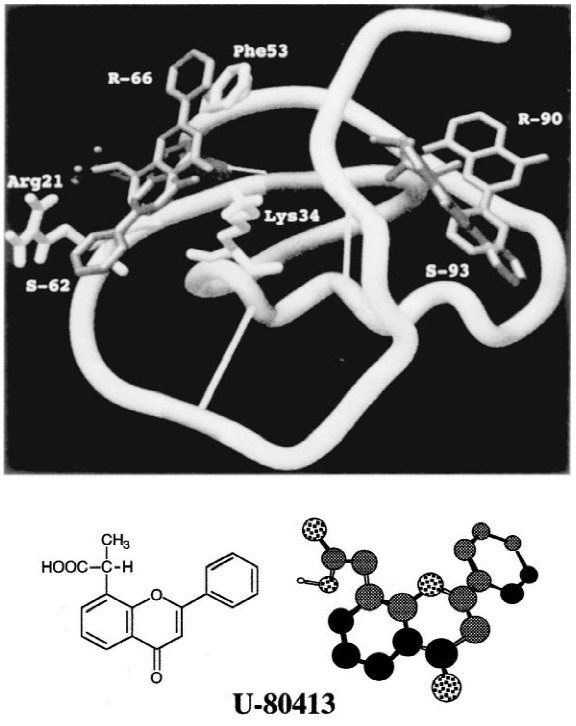

Fig. 8. Molecular modeling simulation of U-80413 enantiomers bound to OMTKY3. Reproduced, with permission, from Ref. [21]. Ligands are labeled according to their *R*- or *S*-chirality and numbered according to their position among the 100 lowest energy minimized binding orientations.

interactions with the positive charge on Arg 21. The carbonyl group on U-80413's central ring shares a hydrogen bond with the NH_3^+ group of Lys 34. The

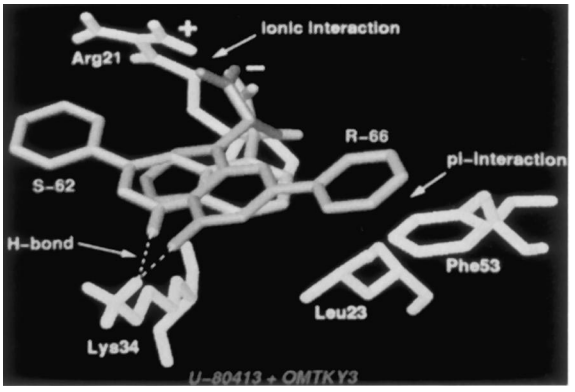

Fig. 9. Enantiomers of U-80413 in the enantioselective binding site of OMTKY3. Reproduced, with permission, from Ref. [21].

distinguishing difference between the enantiomers is the proximity of the phenyl group of (*R*)-U-80413 and Phe 53. However, neither the first, second nor a combination domain of OMTKY gave an appreciable chiral recognition ability [21].

These results may suggest that three domains work in concert for chiral recognition of various solutes, because columns made with the whole, intact OMTKY [79] as well as OMCHI [72,73,75,76] can resolve a wide range of weakly acidic, weakly basic and neutral racemates. The presence of other protein portions in OMCHI preparations was shown by other investigators [80,81]. Recently, Haginaka et al. isolated and characterized a new protein from chicken egg whites [7]. It was termed OGCHI as reported by Ketterer [82]. The only discrepancy of the protein isolated by Haginaka et al. from that isolated by Ketterer was the average molecular mass, which was 30 000 and 24 000, respectively. Perhaps the two proteins are the same. In addition, it was found that 10% of OGCHI was included in crude OMCHI preparations [7]. Table 3 shows a comparison of the chiral recognition ability of crude OMCHI and OGCHI columns. The OGCHI column gave a much more excellent chiral recognition ability than the crude OMCHI column. Moreover, the OMCHI and OGCHI columns were made from isolated, pure proteins and were compared with regard to chiral recognition abilities. It was found [7] that the chiral recognition ability of OMCHI reported previously [6] came from OGCHI, and that OMCHI had no appreciable chiral recognition ability. Further, it was found that OGCHI is preferentially bound to DSC-activated aminopropyl-silica gels compared with OMCHI, despite the average molecular masses of OGCHI and OMCHI being 30 000 and 27 000, respectively [83]. This is why CSPs based on crude OMCHI, which are now commercially available, show moderate chiral recognition ability.

One might ask why whole, intact OMTKY and OMCHI have no chiral recognition ability, but the third domains of OMTKY and OMCHI have. Racemic solutes cannot reach the chiral binding sites on whole, intact OMTKY and OMCHI because of steric hindrance, but can reach the chiral binding sites on the third domains after cleavage of the connection peptide of each domain.

The effects of sugar moieties of OGCHI on the

Table 3
Comparison of retention factors (k_1), enantioselectivity (α), and resolution (R_s) of various solutes on columns made with crude OMCHI and isolated OGCHI.[a] Reproduced, with permission, from Ref. [7]

Compound	Column					
	Crude OMCHI			OGCHI		
	k_1	α	R_s	k_1	α	R_s
Benzoin	2.50	2.71	6.06	11.4	3.18	10.1
Hexobarbital	0.35	1.00		1.52	1.29	0.83
Alprenolol	2.53	1.12	0.31	15.9	1.13	0.84
Propranolol	7.49	1.12	0.44	42.6	1.18	0.78
Chlorpheniramine	1.03	2.05	3.00	5.42	2.27	5.89
Ibuprofen	4.05	1.18	0.88	9.03	1.39	2.58
Ketoprofen	7.69	1.11	0.82	23.5	1.20	1.97

[a] HPLC conditions: column, 2.0 mm I.D.×100 mm; eluent, 20 m*M* phosphate buffer (pH 5.1)–ethanol, 90:10 (v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 220 nm.

enantioseparations of various solutes were investigated. After removal of sialic acid and sialic acid-galactose groups of OGCHI by treatment with neuraminidase and neuraminidase-galactosidase, respectively, CSPs based on desialyl OGCHI (asialo OGCHI) and desialyl-degalactosyl OGCHI (asialo-agalacto OGCHI) were prepared [84]. The retentive and enantioselective properties of OGCHI, asialo OGCHI and asialo-agalacto OGCHI columns were compared. Generally, removal of a sialic acid or sialic acid-galactose group of OGCHI resulted in no change or an increase of the retention factors and enantioseparation factors of the solutes tested. It was concluded that sialic acid and sialic acid-galactose groups of OGCHI do not participate in chiral discrimination of the solutes tested [84]. Moreover, pd-OGCHI and completely deglycosylated OGCHI (cd-OGCHI) were obtained by treatments of OGCHI with *N*-glycosidase, and a mixture of endoglycosidase and *N*-glycosidase, respectively [85]. The average molecular masses of pd-OGCHI and cd-OGCHI were estimated to be about 28 400 and 21 400, respectively. Although OGCHI has five sugar chains, one sugar chain might be cleaved by *N*-glycosidase. It was found that the pd-OGCHI column showed excellent chiral recognition ability, comparable to the OGCHI column, and that the retentivity and enantioselectivity of basic solutes tested on the pd-OGCHI column were higher than those on the OGCHI column, while those of acidic solutes on the pd-OGCHI column were lower. Further, cd-OGCHI still showed chiral recognition ability for the various solutes tested. These results revealed that the chiral recognition site(s) for OGCHI existed on the protein domain of OGCHI [85].

OGCHI was bound to aminopropyl-silica gels via an amino or carboxyl group(s) of OGCHI [86]. In the former case, OGCHI was bound to DSC-activated aminopropyl-silica gels, while in the latter case OGCHI activated by a water-soluble carbodiimide and HSSI was bound to aminopropyl-silica gels. The obtained OGCHI materials were compared with regard to the retentivity and enantioselectivity of various solutes. Fig. 10A and B show chromatograms of 2-phenylpropionic acid and naproxen on OGCHI columns via an amino and carboxyl group(s) of the protein, respectively. The OGCHI materials prepared via a carboxyl group(s) of OGCHI are suitable for chiral resolution of acidic solutes, and those prepared via an amino group(s) of OGCHI are suitable for chiral resolution of basic solutes. It was suggested that electrostatic interaction between an amino or carboxyl group of OGCHI and a charged solute should play important role in chiral recognition of the solute [86].

The chiral recognition properties of OGCHI and ovoglycoprotein from Japanese quail egg whites (OGJPQ) were compared [87]. The average molecular masses of OGCHI and OGJPQ were estimated to be about 30 000 and 27 400. The OGCHI column is suitable for chiral resolution of basic compounds, while the OGJPQ column is suitable for that of

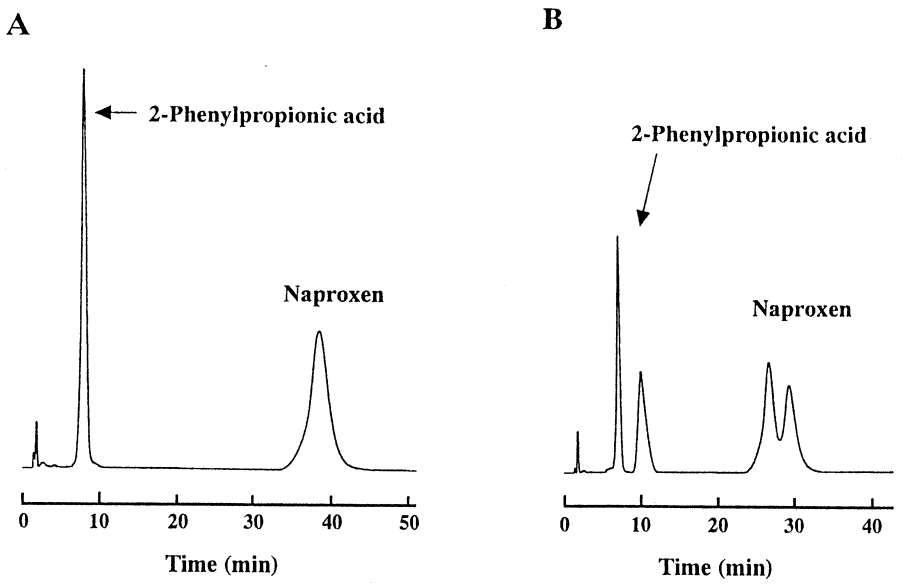

Fig. 10. Chromatograms of 2-phenylpropionic acid and naproxen on OGCHI columns via an amino (A) and carboxyl group(s) (B) of the protein. Reproduced, with permission, from Ref. [86]. HPLC conditions: column, 2.0 mm I.D.×100 mm; eluent, 20 m*M* phosphate buffer (pH 4.0)–ethanol (90:10, v/v); column temperature, 25°C; flow-rate, 0.2 ml/min; detection, 220 nm.

acidic compounds. With regard to chiral resolution of neutral compounds, it is dependent on the compound resolved which column is most suitable. Differences in the retentivity and enantioselectivity between OGCHI and OGJPQ columns are due to differences in the enantioselective binding properties [87]. The results obtained revealed that chiral recognition of various solutes should be efficiently attained using both columns complementarily.

3.2.3. Avidin

AVI is a basic glycoprotein, with a p*I* of 10.0, from egg whites, and strongly binds biotin with an association constant of $\sim 10^{15}\ M^{-1}$ [88]. Miwa et al. immobilized AVI to DSC-activated aminopropyl-silica gels and applied it to the enantioseparation of acidic compounds such as 2-arylpropionic acid derivatives [8]. In addition, AVI was immobilized to aminopropyl-silica gels activated with DSS [33]. The column was utilized for the resolution of acidic, basic and neutral compounds [34,89], and further used for direct serum injection assays of drug enantiomers in biological fluids [33,90]. The hydrophobic spacer linking avidin to the aminopropyl-silica materials significantly affected the retention and separation of drug enantiomers on the AVI column [33,34]. This could be due to the fact that a sufficiently long spacer relieves any steric restrictions imposed by the matrix backbone, thus allowing increased flexibility and availability of the ligand [34].

It is interesting to examine the effect of biotin binding to an AVI column on the retentivity and enantioselectivity of various solutes. Not only enantioselectivity, but also the interaction with racemates, was significantly lost by the formation of the AVI–biotin complex [7,34]. There are two possible explanations for the effects of biotin on the retentivity and enantioselectivity of solutes. One is that some solutes have a high affinity for the biotin binding sites. The other is that the strong interaction between AVI and biotin may cause a conformational change. Further, Oda et al. prepared modified AVI columns by acylation of amino and carboxyl groups [91]. They found that these functional groups contributed to chiral recognition of some drugs on the AVI column, and that the AVI column had multiple binding sites for chiral separation. Further studies are required to

clarify the chiral recognition site(s) and chiral recognition mechanism on AVI.

3.2.4. Riboflavin binding protein

Mano et al. reported CSPs based on RfBP from chicken egg white for the enantioseparation of acidic, basic and neutral compounds [9]. On the other hand, Massolini et al. [92] described the use of RfBP from chicken egg yolk as the CSP. Egg white and yolk RfBPs appear to be the product of the same gene, but have undergone different post-translational modifications [93]. The amino acid sequence is the same, but it is between 11 and 13 amino acids shorter in the case of yolk RfBP [93]. There are differences in carbohydrates linked between egg yolk and white RfBPs. Both CSPs based on chicken egg yolk and white RfBPs gave similar enantioselectivity. Recently, CSPs based on quail egg white RfBP were developed by Lorenzi et al. [94].

The chiral recognition sites and mechanism of RfBP were investigated using capillary electrophoresis (CE) [95]. It was assumed that the α-helix structure region of RfBP could play an important role in chiral recognition of ketoprofen, and that the critical groups involved could be a tryptophan residue, an amino group, and a carboxyl group of the protein. With an increase in the methanol content, the enantioselectivity decreased by more than 25%. This could be due to the increase in methanol content in the running buffer resulting in changes of the α-helical structure of the chiral recognition region, decreasing the chiral recognition capacity. Further, at more than 25% methanol, the α-helical structure of the chiral recognition region is denatured, and the chiral recognition ability may be lost. The authors concluded that the retention behavior in HPLC was not the same as in affinity CE, because non-specific interactions of the solute with the silica gel surface, unreacted aminopropyl groups and spacer linkages contribute to the retention changes in HPLC [95].

3.3. Chiral stationary phases based on enzymes

3.3.1. Trypsin and α-chymotrypsin

Both trypsin and α-chymotrypsin are a family of serine proteases, and catalyze the enantioselective hydrolysis of amides and esters. CSPs based on trypsin and α-chymotrypsin were introduced by Thelohan et al. [10] and Wainer et al. [11], respectively. Trypsin-based CSPs can resolve O- and N,O-derivatized amino acids which are substrates of the enzyme [10]. This means that chiral separations are due to the activity of the enzyme, and that the chiral recognition site is on the enzyme activity site. α-Chymotrypsin-based CSPs can resolve amino acids, amino acid derivatives, dipeptides, and other compounds such as naproxen and aryloxypropionic acids [10,29,96].

Different ways of immobilizing α-chymotrypsin via reactive epoxy, aldehyde and tresyl groups have been studied [29]. It was shown that the use of an epoxide silica gave the best immobilization ratio and a good ability of the enzyme for structural recognition, whereas the aldehyde process offered better stability because of some crosslinking during the immobilization reaction. The use of in situ immobilization with tresyl chloride gave good reproducible CSP.

With regard to the chiral recognition mechanism on α-chymotrypsin-based CSP, it was treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which resulted in blocking of the active site of the enzyme. The obtained TPCK-α-chymotrypsin-based CSP was unable to resolve stereochemically most of the substrates separated on the native α-chymotrypsin-based CSP, although some enantiomeric amino acid esters were separated with higher stereoselectivity. The results suggest that binding interactions between dipeptides and the α-chymotrypsin column mainly occur at the active site of α-chymotrypsin and at other hydrophobic sites on α-chymotrypsin [96]. Recently, Felix and Descorps [97] investigated the recognition mechanism of a solute on α-chymotrypsin-based CSPs, and found that the retention, efficiency and stereoselectivity of the solute appear to be related to their molecular structure, hydrophobicity and electrostatic interactions. Those relationships determine the recognition mechanism and the position of each enantiomer in the enzymatically active site, which is formed by His 57, Asp 102, Gly 193, Ser 195 and Ser 214 [98].

3.3.2. Cellulase

The term cellulase means that there are several enzyme families with quite different structures, but with the common ability to hydrolyze β-1,4-

glycosidic bonds. The fungus *Trichoderma reesei* produces four major cellulases, two cellobiohydrolases, CBH I (64 k, p*I* 3.9) and CBH II (53 k, p*I* 5.9), and two endoglucanases, EG I (55 k, p*I* 4.5) and EG II (48 k, p*I* 5.5) [99]. They are all acidic glycoproteins and have a common structural organization with a binding domain connected to the rest of the enzyme (i.e., the core) through a flexible arm [100]. The interconnecting region is rich in Ser, Thr and Pro residues and is highly glycosylated [100]. The core is enzymatically active. CSPs based on CBH I, which can resolve acidic, basic and uncharged racemates into their enantiomers, have been most extensively investigated among CSPs based on cellulases [99]. Especially, higher enantioselectivity was obtained for the separation of β-blocking agents such as propranolol, oxprenolol and metoprolol [101]. Contrary to CBH I, CBH II could separate only a few β-blockers, but some racemates, e.g. mexiletine, ibuprofen, chlorthalidone and pentobarbital, could be separated [102].

It is interesting that, on the CBH I column at eluent pH 5.5, the retention time of the less-retained (*R*)-propranolol enantiomer decreases with increasing temperature, while the retention time of the *S*-enantiomer increases, causing a large increase of the enantioseparation factor when the temperature is raised from 5 to 45°C, as shown in Fig. 11 [99]. From thermodynamic studies [103], it was found that

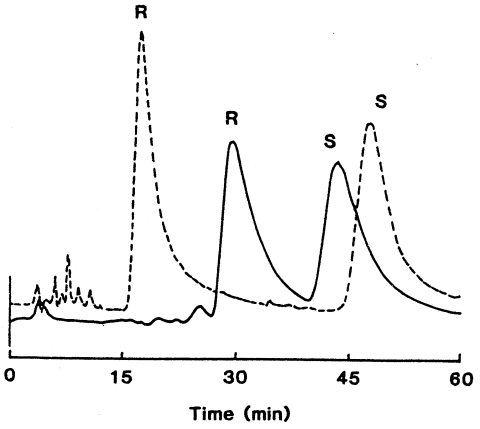

Fig. 11. Separation of racemic propranolol at 10 and 45°C. Reproduced, with permission, from Ref. [99]. Mobile phase: acetate buffer pH 5.5, $I = 0.02$. Straight and dotted lines indicate chromatograms obtained at 10 and 45°C, respectively.

the interaction enthalpy and entropy on non-specific binding sites was −1.1 kcal/mol and +0.1 cal/(mol K), respectively. For specific binding sites, the values were −1.9 kcal/mol and −2.6 cal/(mol K), respectively, for (*R*)-propranolol and +1.6 kcal/mol and +11.6 cal/(mol K), respectively, for (*S*)-propranolol. This is why the unusual chiral separation of propranolol on CBH I occurs. Hedeland et al. [104] reported that a temperature rise from 15 to 25°C gave no significant difference in the circular dichroism (CD) spectra of CBH I, but that at 45°C the CD spectra indicated a conformational change of the protein. Further, an increase in both retention and enantioselectivity for some β-blockers was observed when exchanging potassium for sodium ion in the buffer used as the eluent [104]. The CD spectra of CBH I dissolved in potassium or sodium phosphate buffer display a small difference. This can be explained by an altered protein conformation, which might alter the retention behavior as well as produce a different enantioselectivity for β-blockers.

CBH I was degraded enzymatically into two fragments (core, and flexible region+binding domain) by Marle et al. [20]. Fig. 12 shows the enantioseparation of propranolol on the CSPs based on intact CBH I and the two fragments. Each fragment was shown to contain at least one enantioselective site for propranolol, while the flexible region+binding domain had much lower affinity. The dominating enantioselective site of propranolol and other solutes is located on the core, the main part of the enzyme [20]. Compared to the intact protein, the core fragment showed a higher enantioseparation factor for propranolol, but a significantly smaller retention factor, indicating the absence of non-stereospecific sites in the core fragment [20]. CBH II was also degraded enzymatically into two fragments [102]. The dominating enantioselective site of the solutes is located on the core.

Recently, the three-dimensional structure of the enzymatically active site of CBH I was elucidated by X-ray crystallography [105,106]. It has been shown that the binding site is a ~40 Å long tunnel, as shown in Fig. 13a [107]. Of the CBH I-mutant CSPs, only D214N retained enantioselectivity, whereas the enantioselectivity was completely lost for E212Q and E217Q columns. The loss of enantioselectivity was accompanied by a loss of catalytic activity for the

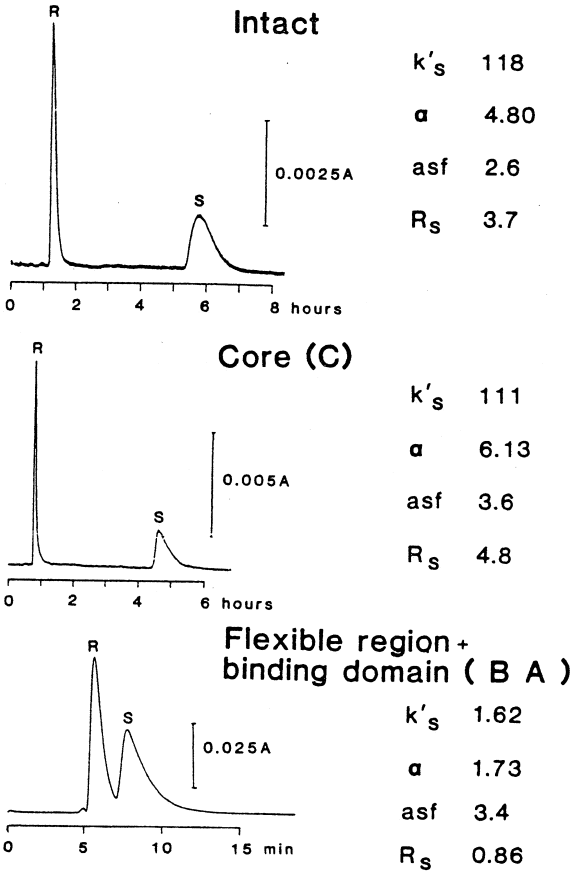

Fig. 12. Separation of the enantiomers of racemic propranolol at pH 6.8 on intact and fragmented CBH I columns. Reproduced, with permission, from Ref. [20]. Mobile phase: 0.065 *M* 2-propanol in phosphate buffer. Loaded amount: 1.0 nmol.

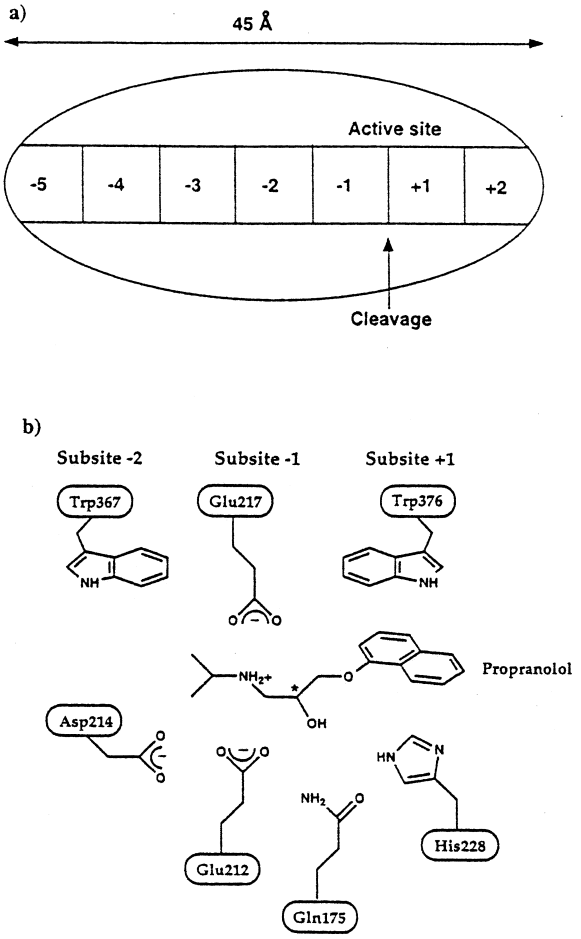

Fig. 13. Schematic representation of the cellulose-binding tunnel in CBH I (a) and schematic model for the binding of propranolol in the active site of CBH I (b). Reproduced, with permission, from Ref. [107]. The tunnel is ~45 Å long and contains at least seven subsites for binding of glucosyl residues. The active site, where glycosidic bond cleavage occurs, is located near the exit of the tunnel between subsites −1 and +1. In (b), the amino acid residues in or near the active site are shown.

mutants, which was determined from kinetic experiments using oligosaccharides as the substrates. These results revealed that carboxylate functions of Glu 212 and Glu 217 were essential for catalysis as well as chiral recognition, and exchange of either one impaired both the activity and chiral recognition. Asp 214 appears to be involved in the chiral recognition, but is of less importance. Thus, the carboxylate groups of Glu 212 and Glu 217 interact with the nitrogen group of propranolol, and Try 376 interacts with the naphthyl moiety (Fig. 13b) [107]. The drastic improvement of enantioselectivity at higher pH indicates that, in addition to the tunnel, deprotonation of one or more of the active site carboxylates is required for binding of β-blockers and to obtain high resolution in chiral separation. Further, these results were confirmed with covalent modification of the carboxylic groups of CBH I [108]. The covalent modification was carried out either in the presence or absence of cellobiose, which has been demonstrated to inhibit the enzymatic activity, and, if present in the mobile phase, impairs the enantioselectivity of β-blockers. Compared to the reference unmodified CBH I column, the covalently modified CSPs showed differences in both enantio-

selectivity and retention. The enzymatic differences between the CSPs were also in line with the chromatographic results. The enantioseparation factors of propranolol were almost unchanged during the reaction period in the presence of cellobiose, while they decreased rapidly without the inhibitor.

3.3.3. Lysozyme

CSPs based on lysozyme were developed by Haginaka et al. [13]. Lysozyme was covalently bound to aminopropyl-silica gels using the DSC-activation reaction. By using a mixture of phosphate buffer and organic modifier as eluent, basic and uncharged enantiomers were resolved, while no resolution of acidic enantiomers was observed.

3.3.4. Pepsin

CSPs based on pepsin were introduced by Haginaka et al. [14]. The pepsin-based CSP could separate basic and uncharged enantiomers, while no resolution of acidic enantiomers was observed [14]. When an eluent of pH 7 was continuously delivered, the pepsin column lost its chiral recognition properties [14]. It has been reported that pepsin is denatured at pH 8.5 [109]. These results reveal that the immobilized pepsin should be irreversibly denatured above pH 7. Further, pepsin and OMCHI were mixed-immobilized onto the same porous aminopropyl-silica gels [110]. The retentive and enantioselective properties of the mixed-protein-based CSP were compared with those of pepsin- and OMCHI-based CSPs. The pepsin-OMCHI-based CSP showed a similar enantioselectivity to the pepsin-based CSP because OMCHI has no enantioselectivity. In addition, the pepsin-OMCHI-based CSP was more stable than the pepsin-based CSP for repetitive injections of samples and continuous flow of the eluent. The pepsin-OMCHI-based CSP was further stabilized by crosslinking with glutaraldehyde [110].

3.3.5. Amyloglucosidase

Recently, CSPs based on amyloglucosidase were introduced by Karlsson and coworkers [15,111]. Several racemic β-blockers were separated into their enantiomers using this CSP. The protein was immobilized to aldehyde silica, which is obtained by oxidation of diol silica via reductive amination. Diol silicas with three different pore sizes, 300, 500 and 1000 Å, were studied in order to examine the amount of enzyme immobilized and the enantioselectivity [15]. The amount of protein immobilized increased in the order 300 Å$<$1000 Å$<$500 Å. The different amounts of protein immobilized to the silica surface may be caused by the density of aldehyde functions as well as the portion of pores, i.e. the specific inner pore surface. The 500 Å diol silica gave the highest enantioselectivity and stability. High enantioseparation factors and symmetrical peaks with column efficiencies of up to 25 000 plates/m were obtained [15].

The enantioselective retention could be controlled by several mobile-phase parameters, e.g. type and concentration of organic modifier, mobile-phase pH, ionic strength and column temperature [111]. A high mobile-phase pH ($>$6) favors enantioselective resolution of the tested solutes. For several of the β-blockers the analysis time could be shortened by increasing the buffer ionic strength without sacrificing the enantioselective resolution. The highest enantioseparation factors were obtained using 2-propanol and, interestingly, an increase in the organic modifier concentration resulted in increased retention and enantioselectivity. Thermodynamic studies showed that the chiral discrimination at a low 2-propanol concentration was entropy-driven, while enantioselective retention was enthalpy-driven at higher 2-propanol concentrations [111]. The effects of column temperature and concentration of 2-propanol on the enantioseparation of alprenolol are shown in Fig. 14. The drastic increase in enantioseparation on increasing the content of 2-propanol in the mobile phase as well as the increase in enantioselective retention by increasing the column temperature are shown.

3.4. Chiral stationary phases based on other proteins

CSPs based on ovotransferrin (or conalbumin) were developed Mano et al. [16]. The column was utilized for the chiral resolution of a basic compound, azelastine, in bulk drugs [16], and for the assay of enantiomers in biological fluids [112]. Ovotransferrin was stable to heat when combined with iron, copper, manganese and zinc [113]. Fur-

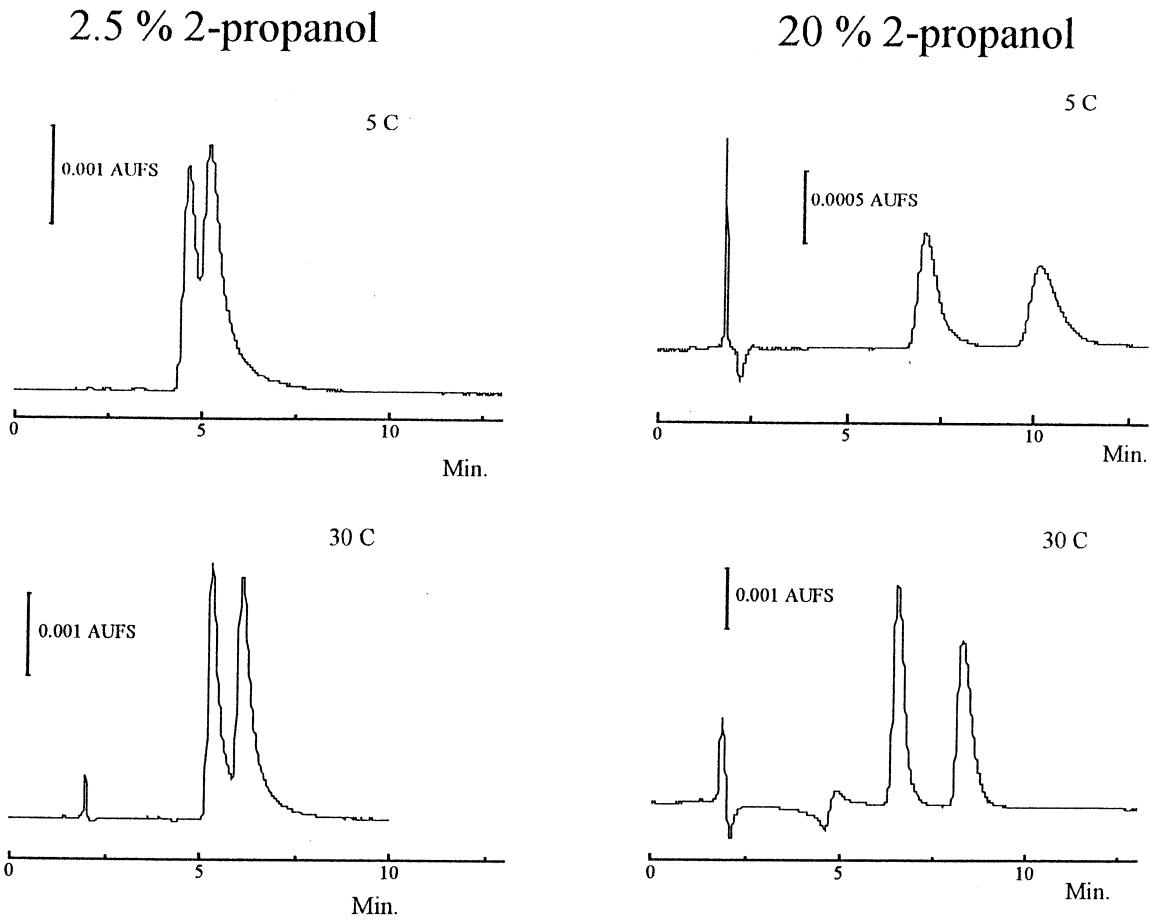

Fig. 14. Effects of 2-propanol and column temperature on the enantiomeric resolution of alprenolol. Reproduced, with permission, from Ref. [111]. Mobile phase: phosphate buffer (pH 7.0, $I = 0.01$) and 2-propanol. Solute: (*R*)- and (*S*)-alprenolol.

ther, ovotransferrin was more stabilized by the conjugation to silica gels as a CSP.

β-Lactoglobulin is quite similar in amino acid sequence and disulfide bond arrangements to members of AGP [17]. The enantioselective properties of β-lactoglobulin as a CSP were examined [17]. However, β-lactoglobulin did not show any enantioselectivity, having only a fairly weak interaction with the majority of the test solutes. Although the protein was examined for use in chiral CE, no solute was enantioseparated. These results suggest that either a specific region which exists on AGP is not present in β-lactoglobulin, or is very significantly modified [17].

4. Conclusions

A variety of proteins have been used for the enantioseparation of various compounds as the chiral selectors in HPLC. The proteins used include albumins such as BSA and HSA, glycoproteins such as AGP, OMCHI, OGCHI, AVI and RfBP, enzymes such as trypsin, α-chymotrypsin, CBH I, lysozyme, pepsin and amyloglucosidase, and other proteins such as ovotransferrin and β-lactoglobulin.

Protein-based CSPs could attain the enantioseparation of a wide range of compounds because of multiple binding interactions and/or multiple binding sites. Various factors, such as eluent pH, type and

concentration of organic modifier and charged modifier, ionic strength and temperature, affect the retentivity and enantioselectivity of solutes on a protein-based CSP. A conformational change of the protein occurs with changes in eluent pH, modifier and temperature. The chiral recognition site(s) of the protein was located and the chiral recognition mechanism was elucidated by NMR measurements, molecular modeling and computational chemistry [21], and X-ray crystallography [51,105]. It was found that hydrophobic, electrostatic and hydrogen bonding interactions were responsible for chiral recognition on protein-based CSPs.

Although CSPs based on a protein fragment or protein domain have been prepared, these CSPs did not always give more excellent chiral recognition abilities than the native ones [19–21]. In the future, a mutant protein, which has different chiral recognition ability and is more stable than the original protein, could be obtained. Further, a protein fragment or protein domain could be prepared by overexpression with genetic technology. It may be possible to make the most suitable chiral selector, based on a protein produced by genetic technology, for the target molecule.

Nomenclature

AGP	α_1-acid glycoprotein
AVI	avidin
BSA	bovine serum albumin
CBH I	cellobiohydrolase I
CBH II	cellobiohydrolase II
cd	completely deglycosylated
CD	circular dichroism
CE	capillary electrophoresis
CSP	chiral stationary phase
DSC	*N*,*N*′-disuccinimidylcarbonate
DSS	*N*,*N*′-disuccinimidylsuberate
EG I	endglycanase I
EG II	endglycanase II
HSA	human serum albumin
HSSI	*N*-hydroxysulfosuccinimide
MALDI-TOF	matrix-assisted laser-desorption ionization time-of-flight
OGCHI	ovoglycoprotein from chicken egg whites
OGJPQ	ovoglycoprotein from Japanese quail egg whites
OMCHI	ovomucoid from chicken egg whites
OMCHI3	unglycosylated third domain of ovomucoid from chicken egg whites
OMCHI3S	glycosylated third domain of ovomucoid from chicken egg whites
OMTKY	ovomucoid from turkey egg whites
OMTKY3	unglycosylated third domain of ovomucoid from turkey egg whites
OMTKY3S	glycosylated third domain of ovomucoid from turkey egg whites
pd	partially deglycosylated
p*I*	isoelectric point
RfBP	riboflavin binding protein
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone

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