Journal of Chromatography, 511 (1990) 89–95
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 400

Characteristics of ovomucoid-conjugated columns in the direct liquid chromatographic resolution of racemic compounds

TOSHINOBU MIWA*, HIROFUMI KURODA and SHIGERU SAKASHITA
Eisai Co., Ltd., Pharmaceutical Research Laboratory, Takehaya machi 1-chome, Kawashima cho, Hashima gun, Gifu 501-61 (Japan)
and
NAOKI ASAKAWA and YASUO MIYAKE
Eisai Co., Ltd., 4th Research Department, 1-3, Tokodai 5-chome, Tsukuba-shi, Ibaragi 300-26 (Japan)
(First received November 29th, 1989; revised manuscript received February 20th, 1990)

ABSTRACT

The chiral recognition properties of ovomucoid-conjugated columns were investigated. Trypsin slightly affected the chiral recognition characteristics of the column. Neuraminidase treatment of ovomucoid columns altered their acidic solute retention properties. Deglycosylated ovomucoid-conjugated columns dit not resolve racemic chlorpheniramine or ketoprofen. A sugar chain is essential for the exibition of chiral recognition ability for ovomucoid.

INTRODUCTION

Recent studies on liquid chromatographic chiral resolution showed that the unique characteristics of chromatography using protein-bonded stationary phases make it a useful analytical method[1]. These columns have been employed for the chiral resolution of a large number of compounds, almost all of which have pharmaceutical importance[1]. The chiral resolution mechanism of proteins is still not clear, although some apparent mechanisms involving racemic solutes and low-molecular-weight ligand-conjugated columns have been proposed[2].

Interest has been shown in the contribution of the physiologically important binding part of proteins to chiral resolution. Albumin [bovine serum albumin (BSA)]-conjugated column[3] and α1-acid glycoprotein columns[4] were developed on the basis of the idea that those serum proteins which take part in enantioselective drug transport *in vivo* must also have a chiral recognition capacity in their immobilized state. However, interacting sites capable of participating in the three-point-holding mechanism of chiral recognition[2] are abundant in large protein molecules, together with a few physiologically active sites. From this point of view, it could be said that almost

0021-9673/90/$03.50

all proteins could be enantioseparators, so that more efforts should be made to find proteins with the property of chiral recognition. We have already reported that egg-white avidin, well known as a biotin acceptor, is capable of chiral recognition of acidic compounds[5]. The chiral resolution capacity of the avidin-conjugated column was strongly affected by biotin, the physiological ligand of the protein. This result strongly suggests that the chiral resolution capacity of a protein is closely related to its physiological effect.

Ovomucoid, which is characterized by a high sugar content and trypsin inhibition, has been used as an enantioselective ligand[1]. The objective of this study was to elucidate the relationship between the physiological effect and the enantioselectivity of ovomucoid. We investigated the chiral recognition properties of ovomucoid-conjugated columns treated with trypsin or neuraminidase, and also prepared a deglycosylated ovomucoid-conjugated column to elucidate the absence of enantioselective activity.

EXPERIMENTAL

Apparatus

A Shimadzu LC-6A pump equipped with an SPD-6A variable-wavelength UV monitor and an SCL-6A automatic sample injector was used. The ovomucoid-conjugated columns used is commercially available as Ultron ES-OVM from Shinwa Kako (Kyoto, Japan). We also prepared an ovomucoid-conjugated column as reported previously[6].

Chemicals

Ketoprofen, *m*-$(C_6H_5CO)C_6H_4CH(CH_3)COOH$, from Nihon Bulk Yakuhin (Osaka, Japan) and chlorpheniramine maleate from Kowa (Nagoya, Japan) were used. Trypsin (type III) and neuraminidase (type X) were purchased from Sigma (St. Louis, MO, U.S.A.). Trifluoromethanesulphonic acid (TFMS) was obtained from Wako (Osaka, Japan). All other chemicals were of analytical-reagent grade or higher quality.

The buffer used in all instances was 20 m*M* potassium phosphate buffer.

Adsorption of trypsin to Ultron ES-OVM and elimination of trypsin from the column.

Trypsin (300 mg) was suspended in 100 ml of the buffer (pH 6.0) at 4°C. This suspension was stored overnight at 4°C, then centrifuged at 3000 *g* for 10 min. The total absorbance of the supernatant was 380 at 280 nm. This solution was passed through an Ultron ES-OVM column (150 mm × 4.6 mm I.D.) at 4°C at a flow-rate of 0.6 ml/min, then the column was washed with the buffer (pH 6.0). The total absorbance recovered in the eluate was 312. The adsorbed trypsin was eluted with a solution of 0.25 *M* sodium chloride in the buffer (pH 6.0).

Enzymatic modification of the ovomucoid-conjugated column by neuraminidase

Ten units of neuraminidase were dissolved in the buffer (pH 5.5) and the resulting solution was recycled at 30°C in the ovomucoid-conjugated column (150 mm × 4.6 mm I.D.) for 4.5 h at a flow-rate of 0.9 ml/min. The recycled solution was passed through an Amicon YM5 filter and sialic acid was determined spectrophotometrically using resorcinol[7].

Chemical deglycosylation of ovomucoid and preparation of deglycosylated ovomucoid-conjugated column

Chemical deglycosylation was performed according to the method of Edge *et al.*[8]. This method was also applied with ovomucoid by Gu *et al.*[9]. Briefly, 12.5 ml of anisole and 25 ml of trifluoromethanesulphonic acid were mixed and cooled to 0°C in ice. Ovomucoid (1 g) was dissolved in the mixture and nitrogen was bubbled into the solution for 2 h with magnetic stirring at 0°C. The reaction mixture was diluted with 75 ml of diethyl ether cooled to −40°C, then 120 ml of ice-cold 50% aqueous pyridine were added. The aqueous phase was washed with diethyl ether, dialysed against water and lyophilized.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 15% acrylamide gel.

The conjugation of deglycosylated ovomucoid to silica gel was performed in the same way as native ovomucoid conjugation[6].

RESULTS

A chromatogram of chlorpheniramine maleate on the Ultron-ES OVM (OVM column) is shown in Fig. 1. The trypsin-adsorbed OVM (Try-OVM) column still exhibited chiral recognition of chlorpheniramine, although its retention capacity decreased markedly (Fig. 2). Desorption of trypsin from the column was performed with 0.25 *M* sodium chloride in the buffer (pH 6.0). The trypsin-desorbed OVM (dTry-OVM) column showed the same retention and separation for chlorpheniramine enantiomers as the OVM column.

The neuraminidase treatment liberated 0.48 mg of sialic acid. The enzyme-treated (NT-OVM) column and the OVM column showed the same retention and

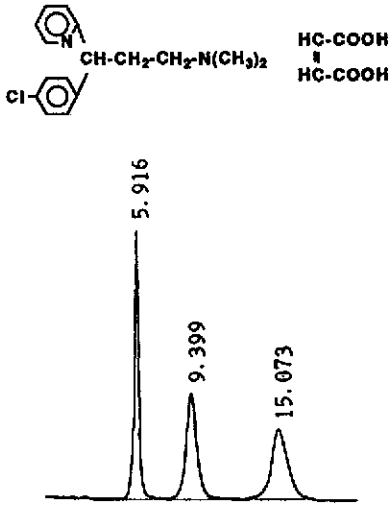

Fig. 1. Chromatogram of chlorpheniramine maleate on the Ultron ES-OVM column. Mobile phase, 20 m*M* potassium phosphate (pH 6.2) containing 12% ethanol; flow-rate, 1.2 ml/min. The elution order is maleic acid, (+)-chlorpheniramine and (−)-chlorpheniramine. Number at peaks indicate retention times in min.

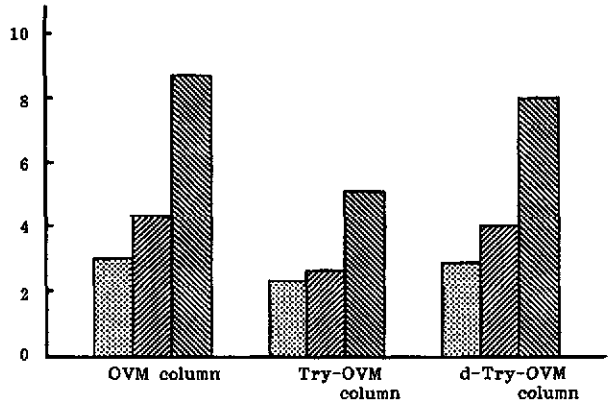

Fig. 2. Retention profile change in the ovomucoid-conjugated (OVM) column by trypsin adsorption. Chlorpheniramine maleate was chromatographed on an OVM columns, a trypsin-adsorbed OVM (Try-OVM) column and the same column trypsin-desorbed (dTry-OVM column). Boxes: left, maleic acid; centre, (+)-chlorpheniramine; right, (−)-chlorpheniramine. The ordinate shows the capacity factor.

separation of racemic chlorpheniramine (Fig. 3); on the other hand, the capacity factor of maleic acid on the NT-OVM column was reduced to 80% of that on the OVM column. Racemic ketoprofen was still resolved by the NT-OVM column, showing two thirds of the capacity factors of the OVM column (Fig. 4).

Chemically deglycosylated ovomucoid migrated faster than native ovomucoid in SDS-PAGE, as shown in Fig. 5. This result coincides with that of Gu *et al.*[9] Deglycosylated ovomucoid was conjugated to succinimide-activated aminopropylsilica gel, which had also been used for the immobilization of native ovomucoid. The effects of the mobile phase pH on the retention of solutes on the deglycosylated ovomucoid-conjugated (dG-OVM) column are shown in Table I. The dG-OVM column did not show chiral recognition of chlorpheniramine and ketoprofen. Chlorpheniramine was strongly retained on the dG-OVM column concurrently with a decrease in the mobile phase pH; this behaviour was the opposite of that of the OVM column.

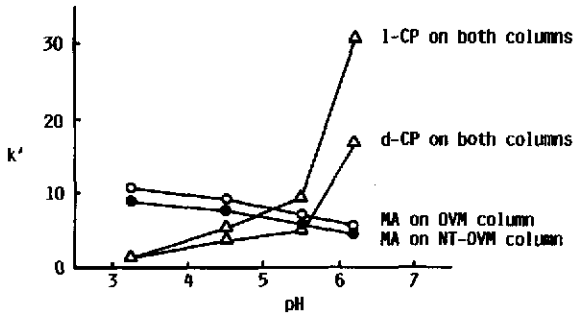

Fig. 3. pH–retention profiles for maleic acid (MA) and (±)-chlorpheniramine (CP) on ovomucoid-conjugated (OVM) column and neuraminidase-treated ovomucoid (NT-OVM) column). ○ = Maleic acid on OVM column; ● = maleic acid on NT-OVM column, △ = chlorpheniramine on OVM and NT-OVM columns. Mobile phase, 20 m*M* potassium phosphate containing 10% ethanol; column temperature, 25°C.

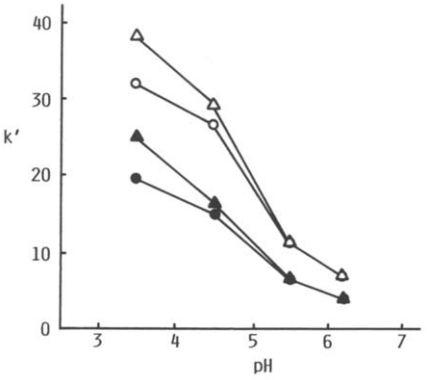

Fig. 4. Influence of pH on the retention of ketoprofen (KP) enantiomers. ○,△ = Ketoprofen enantiomers on ovomucoid-conjugated (OVM) column; ●,▲ = ketoprofen enantiomers on neuraminidase-treated ovomucoid (NT-OVM) column. Mobile phase, 20 m*M* potassium phosphate containing 12% ethanol; column temperature, 25°C.

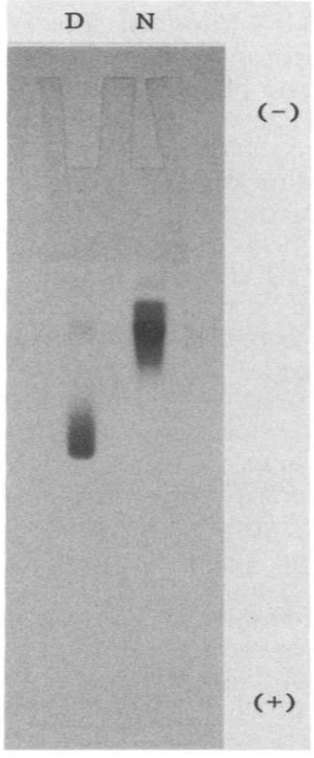

Fig. 5. SDS-PAGE of native ovomucoid (N) and deglycosylated ovomucoid (D). Acrylamide concentration, 15%.

TABLE I

EFFECTS OF pH ON THE RETENTION (k') OF CHLORPHENIRAMINE MALEATE AND KETOPROFEN ON DEGLYCOSYLATED OVOMUCOID-CONJUGATED COLUMN

Racemic resolution of chlorpheniramine and ketoprofen was not achieved. Mobile phase, 20 m*M* potassium phosphate containing 10% ethanol; column temperature, 25°C.

pH	k'		
	Maleic acid	*Chlorpheniramine*	*Ketoprofen*
3.5	0.439	7.23	9.09
4.5	0.602	6.01	12.6
5.5	1.17	4.65	6.80
6.2	1.97	3.66	4.40

The highest capacity for ketoprofen was observed at pH 4.5, but the OVM column retained the compound more strongly at lower pH.

DISCUSSION

Chicken ovomucoid is a triplicate imprinting polymer of a Kazal-type inhibitor, although 1 mol of it inhibits 1 mol of trypsin.[10] Molecular biological studies have shown that ovomucoid is composed of three tandem homologous domains.[11] The trypsin-binding site is located in the second domain, and the proteinase inhibition effects of the first and third domains are almost zero.[11] The objective of this study was to elucidate the chiral recognition properties of proteins, as this may lead to the discovery of new chiral resolution columns. The abundant data on physiological and molecular biological aspects of ovomucoid suggests that this protein could be a suitable material for investigating the chiral recognition properties of proteins.

The chiral recognition of the column was not influenced by trypsin, as Fig. 2 shows. The separation factor (k' ratio) of (±)-chlorpheniramine on this column was not changed by trypsin adsorption. This result suggests that the trypsin-inhibiting site of ovomucoid Arg89–Ala located in domain II was almost unrelated to the differentiation of chlorpheniramine enantiomers. However, the decrease in the capacity factor (k') of solutes on the trypsin-adsorbed column demonstrates that bound ligands could interfere with achiral protein–solute interactions. There are major differences between avidin-conjugated columns and OVM columns regarding the effects of ligands. The chiral resolution ability of the avidin column was eliminated by biotin[5]. The difference between the two columns may be attributed to the K_a values: that for ovomucoid-trypsin is *ca.* $1 \cdot 10^{10}$ l/mol and that of avidin–biotins, $1 \cdot 10^{15}$ l/mol. However, a more important cause of the difference is the conformational change of the proteins, including the sugar chain. We consider that biotin–avidin interaction results in a conformational change, whereas trypsin–ovomucoid interactions does not.

The effect of neuraminidase, which eliminates sialic acid, is interesting. In this experiment, the conjugated ovomucoid was treated with neuraminidase and the chromatographic parameters of maleic acid, ketoprofen and chlorpheniramine were in-

vestigated. The NT-OVM column showed a lower retention of the acidic solutes than the OVM column, although the capacity factor and chiral separation of amines was not affected at all. The chiral resolution of ketoprofen also was not changed. These results imply that the sialic acid of ovomucoid participates in a non-specific retention of acidic solutes. We believe from the results of this experiment that columns with interesting characteristics could be developed even for protein bonded phases by some modification procedure.

Chemical deglycosylation of ovomucoid through TFMS treatment did not alter its trypsin inhibition effect[9], which shows the constancy of its active centre during this treatment. The disappearance of the chiral recognition ability of the dG-OVM column (Table I) indicates that the enantioselectivity of proteins is not attributable to their physiological effect, as we have discussed in relation to the action of trypsin. The pH-retention property of the dG-OVM column for chlorpheniramine and maleic acid was opposite to that of the native OVM column. The dG-OVM column behaves like a counter-ion-exchange column for the above two compounds in the pH range in which these solutes are dissociated. The pH–k' relationship of ketoprofen on the dG-OVM column also suggests the above-mentioned characteristics of the column. The strong hydrophobic interaction which was observed in the OVM column against ketoprofen (Fig. 4) was not recognized in the dG-OVM column. It is clear that the sugar chain of ovomucoid is essential for its enantiospecific interaction with solutes.

REFERENCES

1 S. Allenmark, in A. M. Krstulovic (Editor), *Chiral Separation by HPLC*, Wiley, New York, 1988, p. 285.
2 W. H. Pirkle, T. C. Pochapsky, G. S. Mahler and P. E. Field, *J. Chromatogr.*, 348 (1985) 89.
3 S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 264 (1983) 63.
4 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
5 T. Miwa, T. Miyakawa and Y. Miyake, *J. Chromatogr.*, 457 (1988) 227.
6 T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa and Y. Miyake, *Chem. Pharm. Bull.*, 35 (1987) 682.
7 L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604.
8 A. S. B. Edge, C. R. Faltytnek, L. Hof, L. E. Reichert, Jr., and P. Weber, *Anal. Biochem.*, 118 (1981) 131.
9 J. Gu, T. Matsuda, R. Nakamura, H. Ishiguro, I. Ohkubo, M. Sasaki and N. Takahashi, *J. Biochem.*, 106 (1989) 66.
10 M. B. Rhodes, M. Bennett and R. E. Feeney, *J. Biol. Chem.*, 235 (1960) 1686.
11 I. Kato, J. Schrode, W. J. Kohr and M. Laskowski, Jr., *Biochemistry*, 26 (1987) 193.