Journal of Chromatography, 259 (1983) 154–158
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,545

Note

Affinity chromatography of bile salt 7α-, 7β- and 12α-hydroxysteroid dehydrogenases on immobilized Procion Red and Cibacron Blue Sepharose 4B columns

IAN A. MACDONALD* and YVAN P. ROCHON
Departments of Medicine and Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7 (Canada)
(Received November 18th, 1982)

A number of bacteria in the human intestinal flora elaborate hydroxysteroid dehydrogenases (HSDH) which can oxidize hydroxyl groups of endogenous bile salts to the corresponding ketones[1]. One of the most common bile salt oxidizing enzymes is 7α-HSDH which occurs in *Escherichia coli*[2,3] and *Bacteroides fragilis*[4,5]. Similarly, a 12α-HSDH is elaborated by various non-fermentative clostridia[6,7]. A third common reaction occurring in the human intestine is 7α-hydroxyl epimerization[8,9]. This reaction proceeds via an oxidation of the 7α-hydroxyl group to the corresponding ketone (by 7α-HSDH) and subsequent reduction of the ketone to the 7β-hydroxyl group (by 7β-HSDH)[10–15]. This process can occur within a single organism which elaborates both 7α- and 7β-HSDH (*e.g., Clostridium absonum*[10,11] or lipase and lecithinase-negative clostridia[12,13]) or can ocur in two organisms cocultured together, one elaborating a 7α-HSDH and the second elaborating a 7β-HSDH[14,15]. One such example is *Eubacterium aerofaciens* (7β-HSDH elaborating) which when cocultured with *B. fragilis* (7α-HSDH elaborating) can convert chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid) to ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid, *ca.* 80% yield) via the intermediate 7-ketolithocholic acid (3α-hydroxy-7-oxo-5β-cholanoic acid)[15]. This reaction occurs efficiently even though the *B. fragilis* outnumber the *E. aerofaciens* by more than 100:1.

It should also be mentioned that 7α-HSDH and 12α-HSDH are totally group and stereo specific[3,16] and can be used to quantify these respective groups in bile[17] and fecal[18] extracts. Theoretically 7β-HSDH could be similarly used provided a stable preparation of this enzyme devoid of 7α-HSDH or other interfering enzymes could be obtained. It is the purpose of this paper to describe the use and some of the limitations of triazine dye (Procion Red and Cibacron Blue) affinity columns in the purification of the 7α-, 7β- and 12α-HSDH peparations.

MATERIALS

Crude cell free preparation of *B. fragilis* 7α-HSDH[4], *E. coli* 7α-HSDH[2], *C. absonum* 7α- and 7β-HSDH[19], *E. aerofaciens* 7β-HSDH[15] and *Clostridium* group P 12α-HSDH[7] were prepared as described earlier. All preparations were "shell" frozen and lyophilized in a Vir Tis lyophilizer. Immobilized Procion Red (Reactive Red) and Cibacron Blue (Reactive Blue) Sepharose and EDTA and dithioerythrol (DTE) were obtained from Sigma (St. Louis, MO, U.S.A.). "Econo" glass chromatography

0021-9673/83/0000–0000/$03.00 © 1983 Elsevier Scientific Publishing Company

columns (10 × 1 cm) were from Bio-Rad Labs. (Richmond, CA, U.S.A.). Dibasic and monobasic sodium phosphate were from BDH (Montreal, Canada).

METHODS

Affinity chromatography was performed on a 2 × 1 cm Procion Red or Cibacron Blue column. Approximately 200 mg of lyophilized crude 7α-, 7β- or 12α-HSDH were dissolved in a minimal volume of water (*ca.* 1. ml) and dialyzed overnight against twenty volumes of 0.1 *M* phosphate buffer pH 7.0 containing 1 m*M* of EDTA and DTE. Freshly dialyzed material was placed on the column and allowed to stand for about 15 min, then the column was washed with excess buffer until the absorbance value at 260 nm of the effluent returned to background level (requiring about 150 ml buffer). Enzyme activity was eluted with 600 m*M*, 1.0 *M* or 3.0 *M* NaCl dissolved in column buffer. (See Table I for elution buffer required for each enzyme activity.) Two-ml fractions were collected and monitored at 260 nm and 230 nm and 10–200 μl aliquots were assayed for the appropriate HSDH activity (activities). Fractions of peak enzyme activity were pooled, assayed and protein concentrations were determined with Bio-Rad dye reagent[20]. Aliquots of crude preparations were similarly assayed. Specific activities and fold purification were calculated for each enzyme chromatographed. Purified preparations were dialyzed against twenty volumes of buffer as performed earlier, shell frozen, lyophilized and stored at −20°C.

Scaled up purification procedures were also performed with *C. absonum* 7α- and 7β-HSDH and with *Clostridium* group P 12α-HSDH using Procion Red columns (5 × 1 cm) and 600 mg lyophilized enzyme preparations.

RESULTS AND DISCUSSION

Results are summarized in Table I. Successful purification was obtained for the *C. absonum* 7β- and 7α-HSDH and *E. aerofaciens* 7β-HSDH, and the *Clostridium* group P 12α-HSDH (all NADP-dependent enzymes). In most cases, Procion Red gave better results than Cibacron Blue, both in terms of fold purification and yields. As shown in Fig. 1, 7β-HSDH activity from the mixture of 7α- and 7β-HSDH in *C. absonum* could be selectively eluted from a Procion Red or a Cibacron Blue column by 600 m*M* NaCl. The 600 m*M* NaCl eluate was shown to be devoid of 7α-HSDH activity. Most of the 7α-HSDH could be subsequently eluted by 3.0 *M* NaCl which also contained a portion of 7β-HSDH which did not elute with 600 m*M* NaCl. *E. aerofaciens* 7β-HSDH could be similarly purified by Procion Red chromatogaraphy. Although, in contrast to *C. absonum* 7β-HSDH, this enzyme did not adsorb to the Cibacron Blue column. *Clostridium* group P 12α-HSDH could be conveniently purified by both Procion Red and Cibacron Blue columns, with a markedly higher fold purification with the former. This purification is shown in Fig. 2. In contrast to the NADP-dependent hydroxysteroid dehydrogenases listed in Table I, neither of the NAD-dependent 7α-HSDH enzymes from *E. coli* and *B. fragilis* strongly adsorbed to either affinity system, thus little purification occurred, although the *E. coli* enzyme was slightly retarded on both columns.

In a recent review, Dean and Watson[21] have described a long list of enzymes and proteins which bind to immobilized triazine dyes. It can be noted that none of the above HSDH systems were included in their published list. The commonly used term "affinity chromatography" may in fact be a misnomer. Immobilized dye has been compared to immobilized nucleotide[21]; in each of these systems, however, there

TABLE I

PURIFICATION OF 7α-, 7β- AND 12α-HYDROXYSTEROID DEHYDROGENASES FROM VARIOUS MICROBIAL SOURCES

PR = Procion Red; CB = Cibacron Blue; CDC = chenodeoxycholic acid; chol. = cholic acid; UDC = ursodeoxycholic acid; DC = deoxycholic acid.

Organism	*Enzyme*	*pH*	*Cofactor/ substrate*	*Column*	*NaCl in elution buffer (M)*	*Fold purification*	*Overall yield (%)*	*Comments*
E. coli	7α-HSDH	9.5	NAD/CDC	PR	–	3.8	60.0	Little adsorption
				CB		1.8	80.6	Little adsorption
B. fragilis	7α-HSDH	9.5	NAD/CDC	PR		1.0	80.0	
				CB		1.2	77.7	Little adsorption
C. absonum	7α-HSDH	10.5	NADP/chol.	PR	3.0	8.7	61.6	
				CB	3.0	7.54	86.7	
C. absonum	7β-HSDH	9.5	NADP/UDC	PR	0.60	14	52.7	
					3.0	23.2	16.4	
				CB	0.60	2.46	32.77	
					3.0	3.2	36.86	
E. aerofaciens	7β-HSDH	9.5	NADP/UDC	PR	1.0	6.3	51.0	
				CB	-	1.5	98.1	Little adsorption
Clostridium group P	12α-HSDH	9.5	NADP/DC	PR	1.0	16.1	100.0	
				CB	3.0	11.7	30.06	

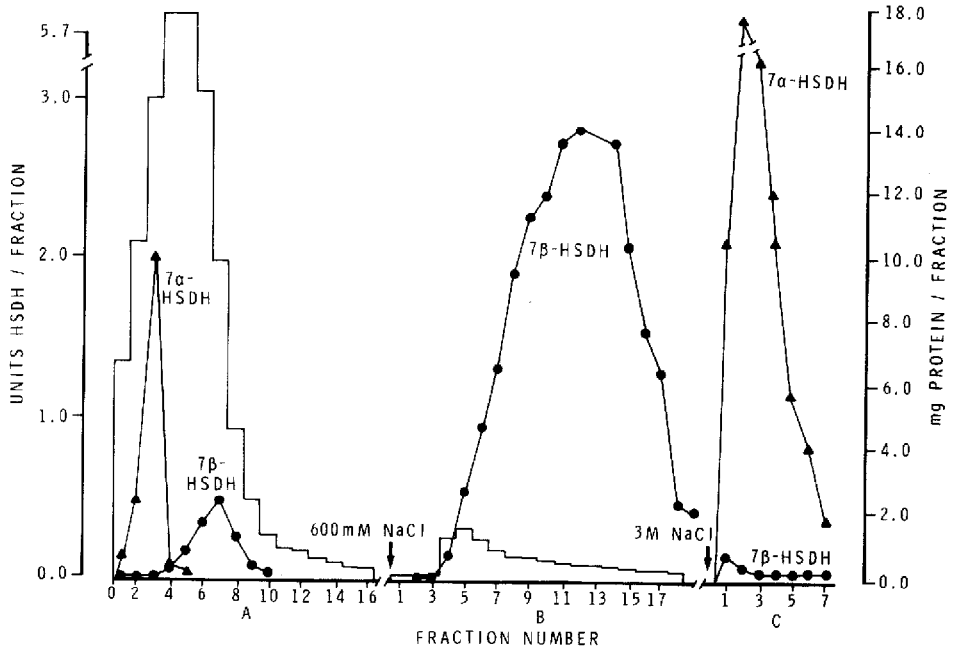

Fig. 1. Immobilized Procion Red affinity chromatography of 7α- (▲- - -▲) and 7β-hydroxysteroid dehydrogenases (●- - -●) from *Clostridium absonum*. Six hundred mg lyophilized powder was chromatographed on a 5 × 1 cm column. Protein per fraction is shown in squared area.

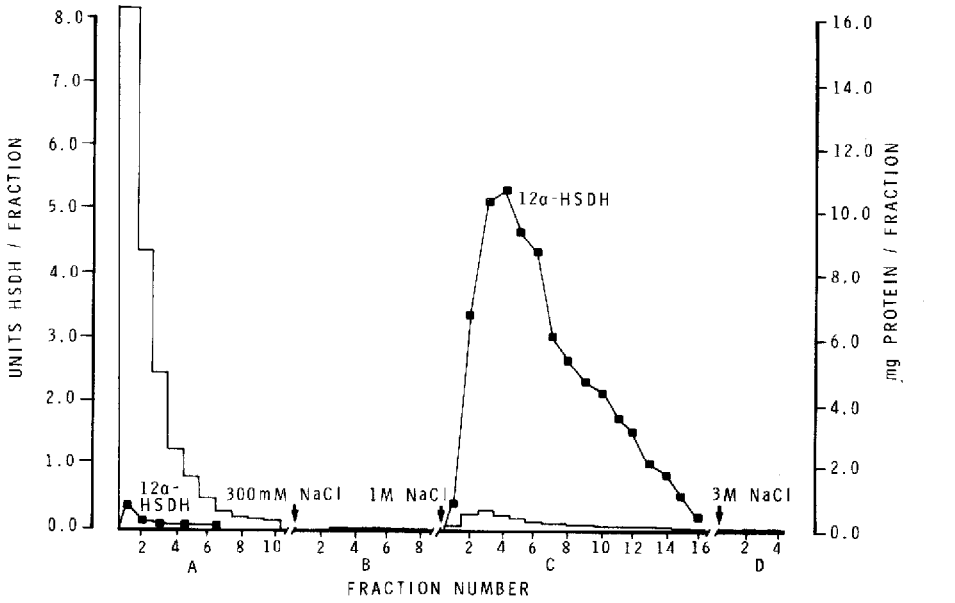

Fig. 2. Immobilized Procion Red affinity chromatography of 12α-hydroxysteroid dehydrogenase (■- - -■) from *Clostridium* group P Strain C4850. Two hundred mg lyophilized powder was chromatographed on a 2 × 1 cm column. Protein per fraction is shown in squared area.

appears to be a lack of specificity both in the mode of binding and elution of proteins[7,21]. None the less, both systems represent powerful purification tools and the immobilized triazine dyes have a much higher degree of substitution and are considerably cheaper than immobilized nucleotide columns[21].

We propose that the above described purification of 7β-HSDH from the *C. absonum* 7α- and 7β-HSDH mixture and that of 12α-HSDH (*Clostridium* group P) may represent a rapid method of generating preparations of these enzymes for the quantitation of 7β- and 12α-hydroxyl groups of bile acids from various biological fluids. Immobilized Procion Red is comparable to immobilized NAD columns for the purification of 12α-HSDH. To our knowledge, no previously described method of cleanly separating the 7α-HSDH component from 7β-HSDH in crude *C. absonum* preparations is available in the literature. The 7β-HSDH in *E. aerofaciens*[22] is far less stable than the 7β-HSDH in *C. absonum*[19]. A slightly lower yield of enzyme activity on Procion Red affinity chromatography occurred in the former (Table I). Additionally the level of crude enzyme activity per culture is also orders of magnitude greater in *C. absonum* when appropriately induced[19], making this organism the source of 7β-HSDH of choice for the quantitation of 7β-hydroxyl groups in biological sources (*e.g.*, bile).

Current studies include the use of Procion Red column purified 7β-HSDH for the estimation of ursodeoxycholic acid in human bile.

ACKNOWLEDGEMENTS

The authors expess their thanks to Drs. P. B. Hylemon and T. Glass of the Department of Microbiology, Medical College of Virginia, Richmond, VA for their scholarly contribution. This work is supported by Grant 5075 from the Medical Research Council of Canada.

REFERENCES

1 S. Hyakawa, *Advan. Lipid Res.*, 11 (1973) 143–192.
2 I. A. Macdonald, C. N. Williams and D. E. Mahony, *Biochim. Biophys. Acta*, 309 (1973) 243–253.
3 E. S. Haslewood and G. A. D. Haslewood, *Biochem. J.*, 157 (1977) 207–210.
4 I. A. Macdonald, C. N. Williams, D. E. Mahony and W. M. Christie, *Biochim. Biophys. Acta*, 384 (1975) 12–24.
5 P. B. Hylemon and J. A. Sherrod, *J. Bacteriol.*, 122 (1975) 418–424.
6 D. E. Mahony, C. E. Meier, I. A. Macdonald and L. V. Holdeman, *Appl. Environ. Microbiol.*, 34 (1977) 419–423.
7 I. A. Macdonald, J. F. Jellett and D. E. Mahony, *Lipid Res.*, 20 (1979) 234–239.
8 T. Fedorowski, G. Salen, G. S. Tint and E. H. Mosbach, *Gastroenterology*, 77 (1974) 1068–1073.
9 S. Hirano, N. Masuda and H. Oda, *J. Lipid Res.*, 22 (1981) 735–743.
10 I. A. Macdonald, D. M. Hutchison and T. P. Forrest, *J. Lipid Res.*, 22 (1981) 458–466.
11 J. D. Sutherland and I. A. Macdonald, *J. Lipid Res.*, 23 (1982) 726–732.
12 R. Edenharder and T. Knaflic, *J. Lipid Res.*, 22 (1981) 652–658.
13 P. Edenharder and H. J. Deser, *Zentrabl. Bakt. Hyg. I. Abt. Org.*, 174 (1981) 91–104.
14 S. Hirano and N. Masuda, *J. Lipid Res.*, 22 (1981) 1060–1068.
15 I. A. Macdonald, Y. P. Rochon, D. M. Hutchison and L. V. Holdeman, *Microbiol.*, 44 (1982) 1187–1195.
16 I. A. Macdonald and F. C. Chang, *Enzyme*, 28 (1982) 392–395.
17 I. A. Macdonald, C. N. Williams and B. C. Musial, *J. Lipid Res.*, 21 (1980) 381–385.
18 M. J. Crowell and I. A. Macdonald, *Clin. Chem.*, 26 (1980) 1298–1300.
19 I. A. Macdonald and P. D. Roach, *Biochim. Biophys. Acta*, 665 (1981) 262–269.
20 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
21 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301–319.
22 S. Hirano and N. Masuda, *Appl. Environ. Microbiol.*, 43 (1982) 1057–1063.