Journal of Chromatography, 177 (1979) 201–208

CHROM. 11,936

CHARGE-TRANSFER* AND WATER-MEDIATED ADSORPTION

III. ADSORPTION ON TRYPTOPHAN-SUBSTITUTED SEPHADEX AND SEPHAROSE

M. A. VIJAYALAKSHMI

Institut de Technologie des Surfaces Actives, Université de Technologie de Compiègne, B.P. 233, 60206 Compiègne (France)

and

JERKER PORATH

Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala (Sweden)

(First received April 2nd, 1979; revised manuscript received April 23rd, 1979)

SUMMARY

The adsorption of tyrosine, tryptophan and some related compounds on tryptophan and tryptophanyltryptophan gels is similar to but much stronger than that observed with the parent gel Sephadex G-25. The adsorption has been attributed mainly to charge transfer or redistribution of electrons and protons leading to π-bonding and hydrogen bonding, in addition to hydrophobic adsorption. The participation of organized water surrounding the gel matrix, the ligands and the solute molecules has been considered.

The application of this principle of adsorption has been exemplified by chromatographic experiments with a cellulase preparation on tryptophan-Sepharose 4B.

INTRODUCTION

After the discovery of the molecular sieving properties of cross-linked dextran (Sephadex®), the existence of other solute–gel interactions were reported in the 1960s[1,2]. The phenomena were later studied in detail by Porath and co-workers[3–5], who suggested that the adsorption might be due to interactions between the π-electrons of the aromatic ring systems of the solutes and the hydrogen or the hydroxyl groups in the matrix or in the gel-bound water.

Further work[6,7] was carried out to enhance these interactions by coupling electron donor or acceptor ligands to these gels. In these investigations the interactions were studied using aromatic amino acids and small peptides.

* "Charge transfer" is used in a broad sense, *i.e.*, including transfer of protons as well as electrons from one atom or group of atoms to another.

As the aromatic amino acids can behave both as donors and as acceptors, we have attempted to couple tryptophan and its dipeptide to the dextran gels and to study their interactions with solutes. This will make possible not only the study of a simple potential separation technique but may also add to our understanding of biological phenomena in which tryptophan or its derivatives are involved.

EXPERIMENTAL

Chemicals

The tryptophan and tryptophan-containing peptides were in the L-form. L-Tryptophan was obtained from Kebo (Stockholm, Sweden) and the model peptides were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland).

Dr. Lars Fägerstam of this Institute kindly supplied us with the cellulase preparations from culture filtrates of *Tricoderma reesi*.

Sephadex G-25 and Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden).

Methods of coupling

Introduction of oxirane groups into Sephadex G-25 and Sepharose 4B was performed in alkaline solution with epichlorohydrin as described previously[7].

About 30 g of the suction-dried epoxy-activated gels were suspended in 40 ml of a 5% solution of L-tryptophan in 2 *M* sodium carbonate solution containing 250 mg of sodium borohydride per 100 ml. The suspension was heated to 60° and kept at this temperature with shaking for *ca.* 24 h. Then the gel was washed abundantly with water and collected over fritted glass and used for analysis.

With ditryptophan, because of its lower solubility, a 2.5% solution of ditryptophan in 2 *M* sodium carbonate solution was used.

The gels thus obtained were colourless, indicating the absence of oxidation products.

Chromatographic techniques

Most of the experiments were carried out on short columns with a total volume about 5.0 ml, as described earlier[8]. A few runs were made on larger columns ($V_T > 20$ ml) when the results of the small column were deemed uncertain. A 20–100-μl volume of sample was applied to the column. The chromatograms were run in 0.1 *M* sodium formate (pH 3), 0.1 *M* ammonium acetate (pH 5) and 0.1 *M* Tris·HCl (pH 8), with or without 4 *M* sodium chloride. In some experiments urea to 4 *M* or ethylene glycol to 50% (v/v) were included in the buffer. The flow-rates were adjusted to about 10 ml/h and 1-ml samples were collected. The effluent from the column was monitored continuously at 280 nm with an LKB Uvicord, Type 4701A, instrument. The experiments were carried out at 4° or 37°.

The cellulase preparation was fractionated on a 25 × 1 cm I.D. column packed with tryptophan-Sepharose 4B in 0.1 *M* ammonium acetate (pH 5). A 0.5-ml sample containing 20 absorbance units (measured at 280 nm) was applied to the column, which was eluted with the equilibrium buffer at a flow-rate of 30 ml/h until no further UV-absorbing material emerged. Fractions of 5.0 ml were collected. The buffer was changed to 0.1 *M* Tris·HCl (pH 8) and the fractions were collected in tubes con-

taining 1.0 ml of 1 *M* ammonium acetate (pH 5) to prevent inactivation (the enzymes are most stable at pH 5.0). The peak fractions were studied by isoelectric focusing.

RESULTS

A series of experiments with suitable model substances were carried out as described. Control experiments were performed under the same conditions using a parent Sephadex G-25 gel of the same batch. The results are summarized in the

TABLE I

DEPENDENCE OF REDUCED ELUTION VOLUMES (V_E/V_T) ON GEL TYPE, pH, SALT CONTENT AND TEMPERATURE

Gel	*Ligand concentration (μmol/g dry gel)*	*pH*	*Molarity of NaCl*	*Temperature (°C)*	*Test substance*			
					Tyr	*Tyr_3*	*Trp*	*Trp_2*
Trp-Sephadex G-25	252	3	0	4	1.40	3.21	2.36	7.52
				37	1.50	2.48	2.10	4.46
			0	4	1.95	15.1	5.17	>20
				37	2.10	11.8	4.04	20
		5	0	4	1.70	2.94	2.58	6.86
				37	1.70	2.55	1.83	3.82
			4	4	1.72	8.04	5.52	>20
				37	1.79	5.23	3.07	>20
		8	0	4	1.64	2.73	2.73	5.96
				37	1.89	2.00	2.33	4.51
			4	4	—	—	—	—
				37	2.25	—	3.37	16.5
Trp_2-Sephadex G-25	143	3	0	4	1.66	2.49	2.43	5.4
				37	2.01	2.01	2.01	3.04
			4	4	2.29	19.0	5.73	>20
				37	—	—	—	—
		5	0	4	2.20	3.18	2.60	7.50
				37	1.90	2.40	1.96	4.17
			4	4	2.16	8.48	5.83	>20
				37	2.45	5.59	3.82	>20
		8	0	4	1.01	1.31	2.62	3.90
				37	—	—	—	—
			4	4	—	—	—	—
				37	2.15	7.25	3.98	—
Sephadex G-25	0	3	0	4	1.18	1.92	1.96	4.25
				37	1.35	1.72	1.80	2.55
			4	4	1.45	4.20	2.85	12.7
				37	1.47	3.92	2.25	8.47
		5	0	4	1.29	1.77	1.87	3.43
				37	1.22	1.47	1.51	2.24
			4	4	1.45	3.25	2.87	9.16
				37	1.33	2.78	2.21	6.13
		8	0	4	1.23	1.63	1.84	2.90
				37	1.24	1.49	1.54	2.27
			4	4	1.31	2.92	2.51	7.65
				37	1.45	2.98	2.35	6.53

TABLE II

DEPENDENCE OF REDUCED ELUTION VOLUME (V_E/V_T) ON SOLVENT COMPOSITION

Gel	*Second solvent component*	*Temperature (°C)*	*pH 3.0*				*pH 5.0*			
			Tyr	*Tyr_3*	*Trp*	*Trp_2*	*Tyr*	*Tyr_3*	*Trp*	*Trp_2*
Trp-	None	4	1.40	3.21	2.36	7.52	1.70	2.94	2.58	6.86
Sephadex		37	1.50	2.48	2.10	4.46	1.70	2.55	1.83	3.82
(252 μmol		4	—	—	—	—	1.02	1.21	1.27	1.80
Trp per	8 *M* urea	37	1.06	1.02	1.11	1.48	0.88	1.19	1.35	1.69
gram dry	50% ethylene	4	1.69	1.95	2.47	2.73	1.75	2.05	2.43	2.95
gel)	glycol	37	1.50	1.53	1.59	1.95	1.40	1.72	1.75	2.08
Trp_2-	None	4	1.66	2.49	2.43	5.45	2.20	3.18	2.60	7.50
Sephadex		37	2.01	2.01	2.01	3.04	1.90	2.40	1.96	4.17
(143 μmol	8 *M* urea	4	—	—	—	—	1.15	1.25	1.38	1.88
Trp per		37	—	—	—	—	1.11	1.28	1.43	1.75
gram dry	50% ethylene	4	1.70	1.78	2.53	2.73	1.84	2.01	2.64	3.59
gel)	glycol	37	1.44	1.41	1.72	1.72	1.58	1.75	1.81	2.16
Sephadex	None	4	1.18	1.92	1.96	4.25	1.29	1.77	1.87	3.45
G-25		37	1.35	1.72	1.80	2.55	1.22	1.47	1.51	2.24
	8 *M* urea	4	1.10	1.18	1.31	—	1.33	1.04	1.26	1.53
		37	0.98	1.0	1.05	1.20	1.07	1.15	1.21	1.43
	50% ethylene	4	1.23	1.25	1.74	1.92	1.19	1.18	1.80	2.20
	glycol	37	0.97	0.93	1.21	1.21	0.99	0.95	1.29	1.33

Tables I–III. The experimental error in the relative elution volumes (V_E/V_T) is estimated to be less than 10%.

Adsorption on the matrix proper

As expected, the matrix itself interacts strongly with aromatic amino acids and peptides. However, the amino acid ligand in tryptophan-Sephadex enhances the adsorption considerably and thus contributes to the mechanism involved. This cooperative effect is even more striking when ditryptophan is used as ligand and/or solute.

Effect of temperature

In all instances where significant temperature effects were observed the adsorption increased upon decreasing the temperature, although the effect was smaller with tyrosine as solute.

TABLE III

REDUCED ELUTION VOLUMES (V_E/V_T) FOR SOME TRYPTOPHAN CONTAINING SUBSTANCES ON Trp-SEPHADEX AT 4° IN 0.1 *M* AMMONIUM ACETATE SOLUTION (pH 5.0)

Test substance	*Molarity of NaCl*	
	0	*4*
Trp	2.32	4.50
Acetyl–Trp	1.92	2.77
Trp–Leu	1.62	4.17
Gly–Trp–Gly–Gly	1.57	2.74

Effect of pH

The adsorption seems to be enhanced at low pH (3.2). For ditryptophan-substituted Sephadex there seems to be a maximum interaction around pH 5.0.

Effect of salt

Salt increases the interaction (Tables I and III). The effect of salt on charge-transfer-dependent adsorption is well established[5,9]. Moreover, the larger the number of aromatic residues the stronger is the influence of salt. We have also observed that the chloride ions but not the monovalent cations tested (K^+, Li^+ and Na^+) affect the interaction.

Effect of denaturants

In 8 *M* urea and 50% ethylene glycol the adsorption is less than in the parent buffer systems. However, adsorption is not abolished in any instance. The effect of temperature is diminished in 8 *M* urea solutions in comparison with the adsorption in parent buffer or in 50% ethylene glycol.

Protein chromatography — a possible field of application?

The cellulase preparation was chosen as a model mixture as it is known to contain two cellulases with different aromatic amino acid contents. Cellulase C_1 contains 6 and 19 and cellulase C_x contains 7 and 27 residues of tryptophan and tyrosine, respectively[10,11].

No enzymatically active substance was adsorbed on the parent gel Sepharose 4B. The protein peak eluted at a position corresponding to $V_E/V_T = 0.7$.

On tryptophan-Sepharose 4B the proteins separated into three peaks (Fig. 1). After the first pigment-containing fraction, C_1 activity was linearly eluted between $V_E/V_T = 3$ and 5 while the migration of C_x was speeded up by the displacement with Tris buffer of pH 8.0. Activity was recovered in the expected regions of the chromatograms when pooled fractions were re-run on the same column.

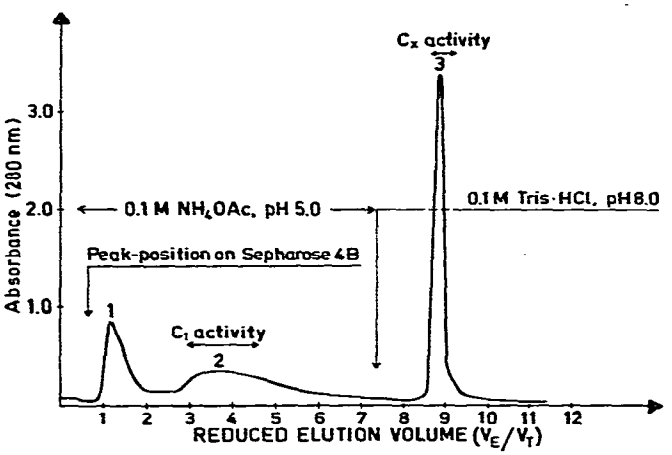

Fig. 1. Chromatogram of a cellulase-containing extract of *Tricoderma reesi*. Adsorbent: tryptophan-Sepharose 4B.

DISCUSSION

We have previously shown that the adsorption of a variety of model substances on charge-transfer adsorbents based on Sephadex is due to a combination of solute–gel and solute–ligand interactions[7,9]. In most instances, the adsorption can be enhanced when the ligand is an electron donor and/or acceptor, as is the case for tryptophan or tryptophan derivatives coupled to the gel. This is shown by the results in Table I, where the adsorption of aromatic substances is generally increased on adsorbents containing tryptophan or tryptophyltryptophan. The increased adsorption is apparently selective, as tryptophan is more strongly adsorbed than tyrosine and ditryptophan is even more strongly adsorbed than trityrosine. In each instance, the extent of adsorption is significantly affected by incorporation of salt in the eluent buffer.

The adsorption phenomenon observed here is not dependent on ionic interaction, as the V_E/V_T values are only slightly pH dependent and are in fact increased in the presence of higher salt concentrations. This might indicate that the adsorption is perhaps due to hydrophobic interaction. However, the decrease in adsorption at 37° compared with that at 4° indicates that hydrophobic interaction cannot be the main cause of the increased adsorption observed here.

These results point to the possibility that matrix-bound water might make a major contribution to the increased adsorption observed. Unequivocal proof of this hypothesis must await extensive future investigations, but the results presented in Table II give some insight in this direction. Incorporation of 8 *M* urea or 50% ethylene glycol in the eluent buffer invariably decreased but did not completely abolish the adsorption (Table II).

It is in fact remarkable that with ditryptophan V_E/V_T is greater than 3 in the presence of 50% ethylene glycol at pH 5 using ditryptophan-Sepharose as adsorbent. The matrix-bound water is thus effectively kept *in situ* and its ordered structure, and consequently its role in the adsorption phenomenon, are not drastically affected by urea or ethylene glycol.

The results in Table III give a further insight into the role played by the structure of the solutes in the adsorption phenomena. Tryptophan is more strongly adsorbed than either acetyltryptophan or tryptophylleucine, indicating that the indole ring system is involved in the interaction in a way which is apparently influenced by the nature of the adjacent amino and carboxylic groups. Incidentally, this result further shows the minor role played by hydrophobic interaction in the overall adsorption phenomena observed. The effect of salt is stronger for the more hydrophobic Trp–Leu than for either acetyltryptophan or Gly–Trp–Gly–Gly.

From the discussion above, it is logical to propose that the major features of the observed adsorption phenomena can be explained, at least partially, by assuming that the solutes, ligands and the gel matrix are surrounded by domains or "water shells" of ordered water structure. The interaction of these domains with one another forms cross-links of the hydrogen-bond type. The strength and lifetime of such bonds depend on the nature of the interacting species and the experimental conditions chosen. The π-electron system of the indole ring may thus act as a proton acceptor and contribute to the stabilization of the 'water shell' around the hydrophobic moiety of tryptophan and tryptophanyl residues. This will lead to stronger

interactions between the "water shells" and thereby increase the extent of adsorption. On the other hand, the "water shells" around tyrosine and trityrosine are presumably less stabilized, with the consequence that these solutes are adsorbed to a lesser extent.

Although this hypothesis reasonably accounts for most of the adsorption phenomena observed, we are still considering the possibility that direct interaction between free and immobilized indole groups may further strengthen adsorption by formation of some kind of π-complex.

The results obtained with the cellulase preparations can be explained, at least in part, by reference to the hypothesis just outlined. In the case of the C_1 enzyme (Fig. 1), the adsorption is apparently linear, indicating that the interaction between the protein molecule and the adsorbent is mediated by water molecules which can be detached easily from the adsorbent surface and the protein solute. This is in contrast to the non-linear adsorption behaviour of proteins with ion exchangers and other adsorbents. The adsorption in such instance is non-linear owing to the phenomenon referred to as "multipoint attachment". The separation of the cellulase enzymes C_1 and C_x on tryptophan–Sepharose is not dependent on the size or charge of the solutes. In fact, their order of migration is the reverse of that found by Håkansson *et al.*[11] when using DEAE-Sepharose, where C_1 was adsorbed while C_x was unadsorbed at pH 5 in the starting buffer. The order of elution observed in Fig. 1 is thus due to an interaction which appears to be tryptophan-dependent, since C_x has a higher content of aromatic amino acids than the C_1 enzyme.

Grushka and collaborators[12,13] have prepared adsorbents by covalent attachment of di- and tripeptides to silica. Such adsorbents are particularily valuable for the rapid fractionation of phenylthiohydantoin derivatives of amino acids. The properties seem to be very different from the adsorbents described in this paper, although tryptophan peptides are strongly adsorbed on both kinds of gels. The polysaccharide gels are more likely to be suited to biopolymer chromatography.

ACKNOWLEDGEMENTS

We thank Drs. D. Eaker, Makonnen Belew, Göran Pettersson and Luis Ochoa for discussions and Dr. Lars Fägerstam for his kind collaboration in the cellulase work. We are grateful to Mrs. I. Johansson and B. Ahlström for expert efficient technical assistance. We also express our thanks to CNRS (France), ITS, Compiègne (France) and NFR (Sweden) for allowing M.A.V. to carry out this work in Uppsala.

REFERENCES

1 J. Porath, *Biochim. Biophys. Acta*, 39 (1960) 193.
2 B. Gelotte, *J. Chromatogr.*, 3 (1960) 330.
3 D. Eaker and J. Porath, *Separ. Sci.*, 2 (1967) 507.
4 J. Porath, *Proc. Nobel Symp.*, 3 (1967) 302.
5 J. Porath, in C. L. A. Harbourn (Editor), *Gas Chromatography 1968*, Institute of Petroleum, London, 1969, p. 201.
6 J. Porath and K. Dahlgren Caldwell, *J. Chromatogr.*, 133 (1977) 180.
7 J. Porath and B. Larsson, *J. Chromatogr.*, 155 (1978) 47.
8 L. E. R. Berghem, L. G. Pettersson and U.-B. Axiö-Fredriksson, *Eur. J. Biochem.*, 53 (1975) 55.

9 J.-M. Egly and J. Porath, *J. Chromatogr.*, 168 (1979) 35.
10 L. Fägerstam and G. Pettersson, *FEBS Lett.*, 98 (1979) 363.
11 U. Håkansson, L. Fägerstam, L. G. Pettersson and L. Andersson, *Biochem. J.*, 179 (1979) in 141.
12 E. Grushka and R. P. W. Scott, *Anal. Chem.*, 45 (1973) 1626.
13 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299.