Journal of Chromatography, 262 (1983) 193–198
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,780

CHARACTERISTICS OF IMMOBILIZED HISTAMINE FOR PYROGEN ADSORPTION

SATOSHI MINOBE*, TADASHI SATO, TETSUYA TOSA and ICHIRO CHIBATA
Department of Biochemistry, Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co. Ltd., 16-89, Kashima-3-chome, Yodogawa-ku, Osaka (Japan)
(Received February 14th, 1983)

SUMMARY

The characteristics of immobilized histamine for pyrogen adsorption were investigated. The adsorbent showed a high affinity for pyrogen at low ionic strength, at around neutral pH, at high temperature and at low flow-rates of a solution containing pyrogen. The adsorption capacity per millilitre of the adsorbent was 0.9 mg pyrogen. Immobilized histamine could be completely regenerated by washing with 0.2 *M* sodium hydroxide solution containing 10–30% ethanol followed by 1.5 *M* sodium chloride solution, or 0.2 *M* sodium hydroxide solution followed by 0.5% sodium deoxycholate solution, 0.2 *M* sodium hydroxide solution and 1.5 *M* sodium chloride solution.

INTRODUCTION

The difficulties in removing pyrogens (lipopolysaccharides, LPS) from certain pharmacologically active substances are well known. A number of attempts have been made to remove pyrogens from certain substances[1–5]. These methods can be applied for removal of pyrogens from relatively stable low-molecular-weight substances, but not from relatively unstable macromolecular substances such as enzymes, hormones and antibodies.

We have tried to prepare adsorbents specific for pyrogens and reported in a previous paper[6] that an adsorbent having histamine as ligand had the highest affinity for pyrogens.

In this paper we describe the characteristics of immobilized histamine for pyrogen adsorption, and the regeneration of this adsorbent.

EXPERIMENTAL

Materials

LPS was purchased from Difco Labs. (Detroit, MI, U.S.A.). Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden), Pre Gel (*Limulus* amoebocyte lysate) from Seikagaku Kogyo (Tokyo, Japan). *tert.*-Butyloxycarbonyl-L-isoleucyl-

0021-9673/83/$03.00

L-glutamylglycyl-L-arginine-4-methylcoumaryl-7-amide (hydrochloride form) (Boc-Ile-Glu-Gly-Arg-MCA-HCl) was purchased from the Peptide Institute (Osaka, Japan), epichlorohydrin, hexamethylenediamine, histamine dihydrochloride and glutaraldehyde from Katayama Chemical Industries (Osaka, Japan) and pyrogen-free water from Tanabe Seiyaku (Osaka, Japan). All other chemicals were analytical reagent grade.

Preparation of immobilized histamine

Histamine was immobilized using aminohexyl-Sepharose CL-4B activated with glutaraldehyde as described previously[6].

Measurement of affinity of adsorbent for pyrogens

The affinity of the adsorbent for pyrogens was measured by a column method as follows. An 8-ml volume of adsorbent was washed with 200 ml of 1.5 *M* sodium chloride solution on a glass filter and then packed in a sterilized column (6.0 × 1.3 cm). The column was washed with 250 ml of 1.5 *M* sodium chloride solution (pyrogen-free) and 100 ml of water (pyrogen-free), and then equilibrated with a solvent described in each experiment. Pyrogen (*Escherichia coli* O128:B12, LPS) was dissolved in the solvent at a concentration of 1000 ng/ml. The pyrogen solution was passed through the column at a flow-rate of 100–500 ml/h at 10–50°C.

Pyrogen assay

Assay of pyrogen was carried out as described previously[6] using Pre Gel and synthetic substrate (Boc-Ile-Glu-Gly-Arg-MCA-HCl).

RESULTS

Adsorption of pyrogen

Effect of salt concentration. The effect of the salt concentration on the adsorption of pyrogen on immobilized histamine was investigated in 0–5.0 *M* sodium chloride solutions. The results are shown in Table I. When the salt concentration was 0–0.5 *M* the affinity of the adsorbent for pyrogen decreased with increasing salt concentration. However, when the salt concentration was higher than 1.5 *M* the affinity increased slightly.

Effect of pH. The effect of pH on the adsorption of pyrogen is shown in Table II. The affinity of the adsorbent was higher at around neutral pH, and decreased at lower or higher pH.

Effect of temperature. The effect of temperature on the adsorption of pyrogen was investigated at various salt concentrations. The results are shown in Table III. At each salt concentration the affinity of the adsorbent for pyrogen increased with increasing temperature, especially in 0.1 *M* sodium chloride solution.

Effect of flow-rate. The effect of the flow-rate of a solution containing pyrogen on the adsorption of pyrogen was tested at flow-rates of 100–500 ml/h. As shown in Fig. 1, the affinity of the adsorbent for pyrogen decreased with increasing flow-rate, especially between 200 and 300 ml/h.

TABLE I

EFFECT OF SALT CONCENTRATION ON ADSORPTION OF PYROGEN ON IMMOBILIZED HISTAMINE

Pyrogen (*E.coli* O128:B12, LPS) was dissolved in 0 5.0 *M* sodium chloride solution at a concentration of 1000 ng/ml. Measurement of the affinity of the adsorbent was carried out as described in the text, using 100 ml of pyrogen solution at a flow-rate of 100 ml/h at 25°C.

Concentration of NaCl (M)	*Concentration of pyrogen in effluent (ng/ml)*
0	0.005
0.05	0.20
0.075	0.85
0.1	9.2
0.15	43
0.2	360
0.3	390
0.5	400
1.0	380
1.5	290
2.0	160
3.0	150
4.0	120
5.0	82

Adsorption capacity

The adsorption capacity of immobilized histamine for pyrogen was investigated as follows. Pyrogen solution (1000 ng/ml) was applied to the column until the pyrogen concentration in the effluent became equal to that of the charged solution,

TABLE II

EFFECT OF pH ON ADSORPTION OF PYROGEN ON IMMOBILIZED HISTAMINE

Pyrogen (*E.coli* O128:B12, LPS) was dissolved (1000 ng/ml) in the following buffers: 7.9 m*M* glycine–hydrochloric acid (pH 3); 10 m*M* sodium acetate (pH 4, 5); 1.15 m*M* disodium hydrogen phosphate 6.6 m*M* sodium dihydrogen phosphate (pH 6); 2.59 m*M* disodium hydrogen phosphate–1.6 m*M* sodium dihydrogen phosphate (pH 7); 10 m*M* sodium 5,5-diethylbarbiturate hydrochloric acid (pH 8, 9); 7.2 m*M* glycine–sodium hydroxide (pH 10); 4.9 m*M* glycine–sodium hydroxide (pH 11). The ionic strength (I = 0.05) was held constant with sodium chloride. Measurement of the affinity of the adsorbent was carried out as described in the text using 100 ml of pyrogen solution at a flow-rate of 100 ml/h at 25°C.

pH	*Concentration of pyrogen in effluent (ng/ml)*
3	92
4	110
5	33
6	2.6
7	3.7
8	19
9	120
10	200
11	570

TABLE III

EFFECT OF TEMPERATURE ON ADSORPTION OF PYROGEN ON IMMOBILIZED HISTAMINE

Pyrogen (*E.coli* O128:B12, LPS) was dissolved in water, 0.05 *M* or 0.1 *M* sodium chloride solution at a concentration of 1000 ng/ml. Measurement of the affinity of the adsorbent was carried out as described in the text, using 100 ml of pyrogen solution at a flow-rate of 100 ml/h at 10–50°C.

Temperature (°C)	*Concentration of pyrogen in effluent (ng/ml)*		
	Water	*0.05 M NaCl*	*0.1 M NaCl*
10	0.064	2.8	140
20	0.006	0.51	9.9
30	0.004	0.42	1.7
50	0.002	0.01	1.1

and the adsorption capacity for pyrogen was determined. As shown in Fig. 2, the adsorbent was slowly saturated with LPS. The adsorption capacity per ml of the adsorbent was calculated to be 0.9 mg for LPS.

Regeneration

About 400 μg of pyrogen were adsorbed on the column packed with 8 ml of immobilized histamine, and the column was washed with various solvents. After washing with 100 ml of water (pyrogen-free) and 100 ml of 0.05 *M* sodium chloride solution (pyrogen-free), 100 ml of pyrogen solution (*E.coli* O128:B12, LPS; 1000 ng/ml in 0.05 *M* sodium chloride solution) were applied to the column and the py-

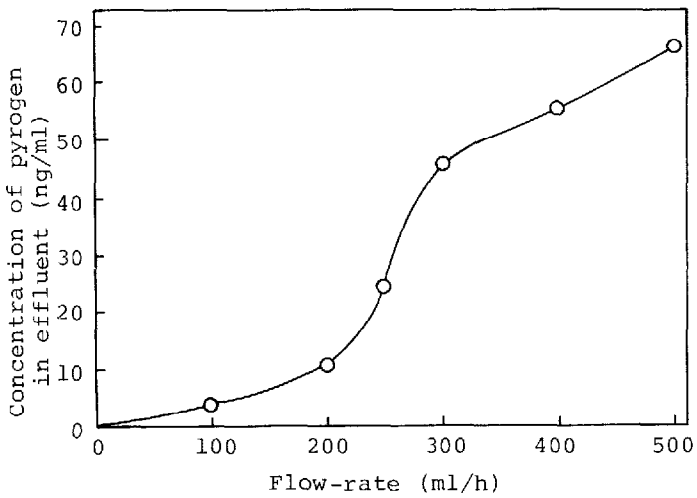

Fig. 1. Effect of flow-rate on adsorption of pyrogen on immobilized histamine. Pyrogen (*E.coli* O128:B12, LPS) was dissolved in 0.05 *M* sodium chloride solution at a concentration of 1000 ng/ml. Measurement of the affinity of the adsorbent was carried out as described in the text, using 8 ml of immobilized histamine and 400 ml of pyrogen solution at flow-rates of 100–500 ml/h at 25°C.

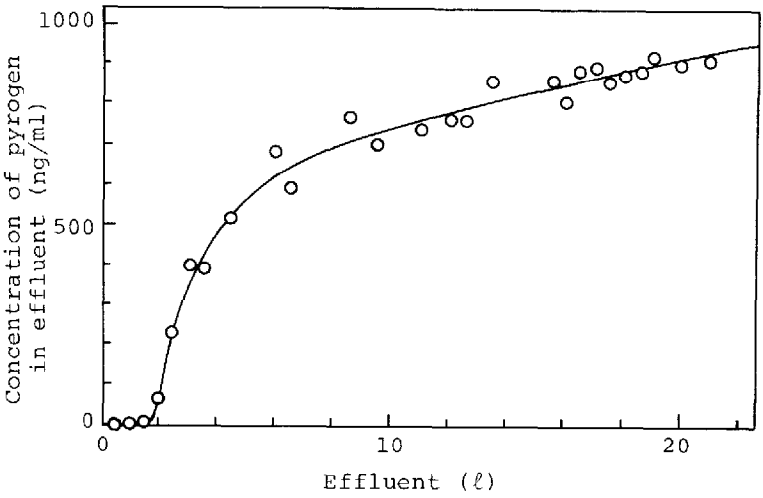

Fig. 2. Adsorption capacity of immobilized histamine for pyrogen. Pyrogen (*E.coli* O128:B12, LPS) was dissolved in 0.05 *M* sodium chloride solution at a concentration of 1000 ng/ml. Measurement of the affinity of the adsorbent was carried out as described in the text, at a flow-rate of 100 ml/h at 25°C. Five hundred millilitres of each fraction were collected, and the concentration of pyrogen in the fractions was determined.

rogen concentration in the effluent was determined. For practical purposes, the pyrogen concentration in the effluent must be less than 1 ng/ml. When the column was washed with 100 ml of 1.5 *M* sodium chloride solution, 0.1 *M* hydrochloric acid solution, 0.2 *M* sodium hydroxide solution, 0.05 *M* sodium hypochlorite solution, 0.05 *M* sodium sulphite solution, 2 *M* guanidine solution, 50% ethylene glycol solution, 50% ethanol solution or 0.1% Triton X-100 solution, respectively, the regeneration of the adsorbent was insufficient. However, the adsorbent could be reused by washing with several solvents: (1) 100 ml of 0.2 *M* sodium hydroxide solution containing 10–30% ethanol followed by 250 ml of 1.5 *M* sodium chloride solution; (2) 16 ml of 0.2 *M* sodium hydroxide solution followed by 32 ml of 0.5% sodium deoxycholate solution, 160 ml of 0.2 *M* sodium hydroxide solution and 250 ml of 1.5 *M* sodium chloride solution.

DISCUSSION

In a previous paper[6] we showed that adenine, cytosine, histamine and histidine as ligands, cellulose and agarose as matrices and spacer chain lengths of 19.7–29.0 Å were the most suitable for the preparation of adsorbents having a high affinity for pyrogen. Moreover the adsorbent prepared by immobilization of histamine on aminohexyl-Sepharose CL-4B with glutaraldehyde treatment had the highest affinity for pyrogen originating from various microorganisms.

We have now investigated the characteristics of the adsorbent for pyrogen adsorption. When the salt concentration was 0–0.5 *M*, the affinity of immobilized histamine for pyrogen decreased with increasing salt concentration (Table I). This suggests that the interaction between the adsorbent and pyrogen could be ionic.

However, when the salt concentration was higher than 1.5 *M*, the affinity increased slightly (Table I). In addition, the affinity increased with increasing temperature (Table III). These results suggest that the interaction is hydrophobic. It is concluded that both ionic and hydrophobic interactions contribute to the adsorption of pyrogen on immobilized histamine.

For practical application, the ease of regeneration is important, and as described in Results the adsorbent could be reused after washing with several solvents.

As shown in Fig. 2, the adsorption capacity per ml of the adsorbent was calculated to be 0.9 mg for LPS. From the results described, this immobilized histamine is considered to be useful for the selective removal of pyrogen in certain biologically active and unstable macromolecular substances. Applications of the method will be described elsewhere.

ACKNOWLEDGEMENT

We are grateful to E. Fukumoto for her technical assistance.

REFERENCES

1 F. Howard and E. C. R. Spoonere, *Chem. Ind. (London)*, (1946) 186.
2 W. E. Smith and R. B. Pennell, *J. Bacteriol.*, 54 (1947) 715.
3 S. Ohkido, *Nippon Kagaku Kaishi*, (1973) 1547.
4 A. R. Martin, *Brit. J. Exp. Pathol.*, 15 (1934) 137.
5 D. H. Campbell and A. Cherkin, *Science*, 102 (1945) 535.
6 S. Minobe, T. Watanabe, T. Sato, T. Tosa and I. Chibata, *J. Chromatogr.*, 248 (1982) 401.