Journal of Chromatography, 298 (1984) 169–171
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,868

Note

Biospecific adsorption of peroxidase

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(First received February 16th, 1984; revised manuscript received May 2nd, 1984)

Hydrophobic adsorption chromatography is a relatively new technique which can be used to purify and characterize proteins[1–5]. This technique has been used to isolate and study several food related enzymes, such as grape and peach polyphenol-oxidase and tomato peroxidase[6–8]. Tomato peroxidase was partially purified using hydrophobic adsorption on phenyl Sepharose[8]. Soybean peroxidase and soybean lipoxygenase were adsorbed on phenyl Sepharose and then separated by differential elution[9,10]. In both of the above studies, binding of peroxidase to phenyl Sepharose was conducted under conditions which promoted hydrophobic interactions.

In this report, tomato and horseradish peroxidase were chromatographed under conditions which promote adsorption on ethyl, butyl, hexyl, octyl, and decyl agarose and on phenyl Sepharose, phenyl agarose, L-phenylalanine agarose and 4-phenylbutylamine agarose. Comparisons between adsorption of peroxidase on the alkyl agarose *versus* adsorption on solid supports containing a phenyl group in the ligand indicated that the binding of peroxidase occurs through biospecific interactions as well as hydrophobic interactions. These results provide new evidence concerning biospecific adsorption of peroxidase onto phenyl Sepharose and agaroses containing an immobilized phenyl group.

MATERIALS AND METHODS

Tomatoes at the breaker stage of maturation were obtained from local commercial sources. Tomato peroxidase was extracted using the procedure described by Jen *et al.*[8]. The isolation of peroxidase included adsorption on phenyl Sepharose, and ion-exchange chromatography on DEAE-Sephacel, followed by preparative electrophoresis. In this investigation, enzyme purified through the DEAE-Sephacel step was used for chromatography on the various adsorbents.

Enzyme assays

Peroxidase activity was determined using 10 m*M* sodium phosphate buffer (pH 6.2), 16 m*M* guaiacol and 1.5 m*M* hydrogen peroxide in a 2-ml assay volume. The increase in absorbance at 470 nm was measured at ambient temperature. One unit of enzyme activity was defined as the amount of enzyme that produced a 1.0-unit change in absorbance per minute at 470 nm. Protein content was determined by the method of Lowry *et al.*[11].

0021-9673/84/$03.00

Chromatography on adsorbents containing immobilized alkyl and phenyl groups

Alkyl agarose chromatography was performed using Shaltiel chromatography kits (Miles Labs., Elkhart, IN, U.S.A.). These kits consisted of a blank agarose control and ethyl, butyl, hexyl, octyl, and decyl hydrocarbon groups bound to agarose. Phenyl Sepharose, phenyl agarose and L-phenylalanine agarose were obtained from Sigma, St. Louis, MO, U.S.A. 4-Phenylbutylamine agarose was obtained from Pierce, Rockford, IL, U.S.A. All columns (1 ml, 1.2 × 0.8 cm) were equilibrated with 20 ml of 100 m*M* sodium phosphate (pH 6.5), containing 2 *M* ammonium sulfate. Both the commercial horseradish peroxidase (ICN Pharmaceutical, Cleveland, OH, U.S.A.) and tomato peroxidase were dissolved in the equilibration buffer. A sample of the enzyme (100 μl; 1–10 μg protein) in 1 ml of the equilibration buffer was applied to each of the columns followed by 15–20 ml of equilibration buffer. The adsorbed enzyme was eluted with 20 ml of water.

RESULTS AND DISCUSSION

Peroxidase was chromatographed on matrices containing immobilized alkyl and phenyl groups using conditions which promoted hydrophobic interactions (*i.e.*, 2 *M* ammonium sulfate). Under these conditions phenyl Sepharose bound tomato and horseradish peroxidase more strongly than the alkyl agarose (Table I). Tomato peroxidase was adsorbed on phenyl Sepharose and 4-phenylbutylamine agarose to a greater extent than on L-phenylalanine agarose, which suggests that the unionized carboxyl group in L-phenylalanine agarose may prevent adsorption. The straight-

TABLE I

ADSORPTION OF PEROXIDASE ON AGAROSES CONTAINING IMMOBILIZED ALKYL AND PHENYL GROUPS

Matrix	*Chain structure*	*Straight-chain equivalent*	*Enzyme bound (%)**	
			Tomato peroxidase	*Horseradish peroxidase*
Agarose		0	0	0
Ethyl agarose	$-NHCH_2CH_3$	C_2	2	2
Butyl agarose	$-NH(CH_2)_3CH_3$	C_4	2	3
Hexyl agarose	$-NH(CH_2)_5CH_3$	C_6	22	3
Octyl agarose	$-NH(CH_2)_7CH_3$	C_8	48	23
Decyl agarose	$-NH(CH_2)_9CH_3$	C_{10}	87	46
Phenyl Sepharose	$-OCH_2CHCH_2OC_6H_5$ (OH)	-**	100	100
L-Phenylalanine agarose	$-NHCHCH_2C_6H_5$ (COO^-)	C_5–C_6	7	0
4-Phenylbutylamine agarose	$-NH(CH_2)_4C_6H_5$	C_7–C_8	90	70

* Enzyme bound percentage was determined as described in the Materials and methods section. Straight-chain equivalent is not known.

** Straight-chain equivalent is not known.

chain methylene group equivalent of phenyl Sepharose is not known, but the hydrophobicity of the benzene ring corresponds to three to four straight-chain methylene groups[12]. Hofstee[1] has indicated that 4-phenylbutylamine agarose and L-phenylalanine agarose are equivalent to seven to eight and five to six straight chain methylene groups respectively. By comparison, the straight-chain equivalent of phenyl Sepharose is probably less than a decyl group. Adsorbents which contained an immobilized phenyl group adsorbed peroxidase more strongly than their straight-chain methylene group equivalent. This would indicate that other interactions in addition to the hydrophobic interactions are responsible for the enhanced binding (compare 4-phenylbutylamine to octyl agarose in Table I).

Many of the substrates for peroxidase are phenyl or phenolic derivatives. This suggests that adsorption of peroxidase to phenyl Sepharose and 4-phenylbutylamine agarose may be due to similarities in structure between the immobilized ligands and substrates of the enzyme. These biospecific interactions may be related to the fit of the immobilized ligand into the active site of the enzyme and result in greater affinity of the enzyme for phenyl groups *versus* alkyl groups. For example, Hofstee[12] has shown that chymotrypsin binds to 4-phenylbutylamine agarose by specific interactions in which the 4-phenylbutylamine ligand behaves as a substrate analogue.

Biospecific adsorption, in addition to hydrophobic adsorption, of a peroxidase onto phenyl Sepharose or 4-phenylbutylamine agarose should be of great use in the isolation of large quantities of peroxidase. The results described here, as well as earlier observations, have shown that this method is rapid, results in high recovery of enzyme, and yields a substantial purification of this enzyme from crude extracts[7–10]. We have not, however, been able to separate different isoenzyme forms using this technique (data not shown). The results described in this paper may have significant application to the isolation of peroxidase by preparative high-performance liquid chromatography (HPLC), since many commercial HPLC columns containing immobilized alkyl and phenyl ligands are now available for hydrophobic and biospecific adsorption.

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