Journal of Chromatography, 317 (1984) 343–347
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 517

ENZYMES ADSORBED ON AN ION EXCHANGER AS A POST-COLUMN REACTOR: APPLICATION TO ACETYLCHOLINE MEASUREMENT

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SUMMARY

Enzymes can be used in high-performance liquid chromatography post-column reactors to improve sensitivity and specificity of detection for some compounds by converting the compounds to easily detectable products. The enzymes can be covalently bound to a post-column reactor, but a simpler approach is to bind them by adsorption to an ion exchanger or a hydrophobic interaction support. This technique has been applied to electrochemical detection of acetylcholine by using adsorption of choline oxidase and cholinesterase to a 3-cm long commercially available weak anion-exchange cartridge. Conversion of acetylcholine to peroxide is quantitative during the 10-sec residence time in the cartridge. Enzyme elution from the cartridge is negligible when low ionic strength mobile phases are used. Fresh enzyme needs to be added to the cartridge at only 1–2 week intervals.

INTRODUCTION

There are many compounds of biological importance which can be separated by high-performance liquid chromatography (HPLC), but not directly detected with useable sensitivity. Pre- and post-column reactions enable measurement of many such compounds. An example is amino acid analysis, which can be achieved by post-column reaction with ninhydrin or *o*-phthalaldehyde, or by pre-column reaction with a wide range of reagents. However, there are compounds which have neither chromophoric nor electroactive nor derivatizable groups. A powerful means of gaining both sensitivity and specificity in detection is to use enzymes to convert the compound of interest to an easily detectable product. This approach has been employed to improve detectability of hydroxysteroids[1–5] or to improve specificity in detection of xanthines and related compounds[6]. In a previous paper[7], an enzyme solution was mixed with the post-column stream to convert acetyl choline (ACh) to betaine and peroxide for electrochemical detection of the latter. Such a system uses a large amount of enzymes, and would be prohibitively expensive with many enzymes. We subsequently developed a method for the routine measurement of ACh which involved preliminary cleanup of tissue extracts by acid, followed by Reineckate precipitation, separation on a polymeric reversed-phase column, conversion of ACh to

peroxide on a post-column enzyme-loaded anion exchange cartridge, and electrochemical detection of the peroxide formed[8]. In the present paper, we describe studies of the factors affecting retention of activity of choline oxidase and cholinesterase on such a post-column reactor system. Immobilization of enzymes by adsorption is a much simpler technique[9] than the covalent coupling techniques used in the hydroxysteroid and xanthine methods.

MATERIALS AND METHODS

Enzymes were purchased from Sigma, St. Louis, MO, U.S.A. The choline oxidase preparation was a lyophilized powder isolated from Alcaligenes sp. The activity was 33 units/mg solid, where 1 unit will catalyse formation of 1 μmol H_2O_2 per min at pH 8 and 37°C. The cholinesterase (Type III from electric eel) was an aqueous solution of 970 units/mg protein, 0.65 mg/ml, where 1 unit catalyses the hydrolysis of 1 μmol/min ACh at pH 8 and 37°C.

The HPLC system used to study retention of enzyme activity consisted of a pulseless high-frequency (23 Hz) reciprocating-plunger pump (Applied Chromatography Systems, State College, PA, U.S.A.) a 6-port sample valve (C6W, Valco, Houston, TX, U.S.A.) and 3 cm × 2.1 mm I.D. Brownlee Aquapore AX300 anion-exchange cartridges (Rainin Inst., Woburn, MA, U.S.A.). Measurement of peroxide formation from Ch and ACh was detected with an electrochemical detector (LC4B, BAS, W. Lafayette, IN, U.S.A.). The oxidation potential of the platinum working electrode was +0.5 V *vs.* a Ag/AgCl reference electrode. Measurement of enzyme elution was made with an Altex/Hitachi variable-wavelength detector. For ACh analysis in tissue extracts, a reversed-phase column is added to the above system ahead of the anion-exchange cartridge[8]. For routine ACh assay, the mobile phase contained 20 m*M* Tris acetate pH 7, 1 m*M* tetramethylammonium chloride, 2% acetonitrile, and 200 μM octane sulfonate.

RESULTS

Enzyme retention vs. salt concentration

Choline oxidase was dissolved in water (2 mg/400 μl). Samples of 5 μl were injected into the AX300 cartridge and chromatographed isocratically at 2 ml/min in 0.4–0.8 *M* solutions of Tris acetate pH 7.5, with detection of proteins at 280 nm (0.05 a.u.f.s.) or of the prosthetic group at 460 nm (0.02 a.u.f.s.). The retention time of the major peak was identical at both wavelengths. The change in retention with salt concentration was quite dramatic. As shown in Fig. 1, there was a 100-fold increase in retention between 0.6 and 0.4 *M*. A mobile phase with a low salt concentration can be chosen, which will cause strong retention of the enzyme on the post-column anion-exchange reactor, and will be compatible with reversed-phase separation of ACh on an analytical column. For 20 m*M* Tris buffer, the extrapolated elution volume of 86 l indicates that retention of enzymes is not likely to be limiting in routine use. However, some salt is necessary, both for buffering action and operation of the electrochemical detector.

Enzyme retention vs. buffer pH

Gradients from 100% water to 100% 1 *M* Tris acetate at the rate of 5%/min

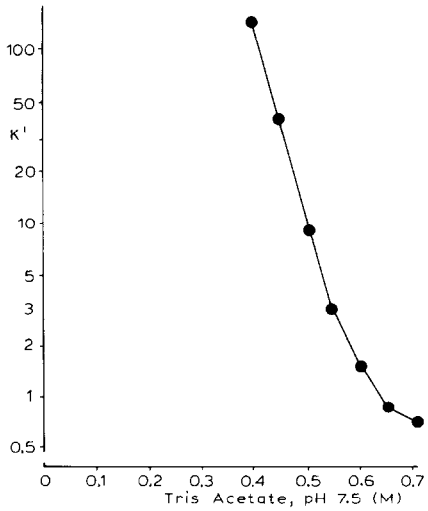

Fig. 1. Retention of choline oxidase on an anion exchanger as a function of salt concentration.

were used with buffers from pH 4.5 to 8.3. The buffer concentration at which choline oxidase was eluted increased from 0.48 *M* at pH 8.3 to 0.86 *M* at pH 4.5. The retention approached a maximum between pH 5.5 and 4.5; lower pH values were not investigated, since there would be no enzyme activity. These retention characteristics are a function both of the isolectric point of the enzyme (pH 4.5)[10], and of the ionization of the stationary phase, which is a mixture of primary, secondary, and tertiary amines with different pK values.

Amount of adsorbed enzyme vs. conversion of substrate

A 20 m*M* Tris acetate buffer, containing 1 m*M* tetramethylammonium chloride, was pumped at 2 ml/min through an AX300 cartridge. Choline oxidase solution (10 mg/ml water) was added to the stream in 20-μl aliquots. After each addition, injections were made of 10 nmol each of choline (Ch) or peroxide. According to the stoichiometry of the reaction, 100% conversion would be indicated by Ch peaks twice that of H_2O_2. Conversion increased from 50% with 0.6 mg choline oxidase to 85% at 1.6 mg to 96% at 2.4 mg. Conversion was quantitative with a total addition of 3 mg enzyme. Conversion of ACh to Ch was then compared after injections of cholinesterase, diluted 1:1 in water. Conversion was 69% of theoretical with 24 μl of enzyme solution, and quantitative with 100 μl. In the absence of tetramethylammonium, there was tailing with Ch and ACh, and the peak heights were lower, although the area was apparently the same. Residence time of the analytes in the column was *ca.* 8 sec. The ability to achieve quantitative reaction in such a short time at concentrations below the 1.2 m*M* K_m[10] is a result of the very large amount of enzyme concentrated in a very small volume.

Enzyme activity vs. pH and time

Six AX300 cartridges were each loaded with 2 mg choline oxidase and 100 μl cholinesterase. The cartridges were then equilibrated with 20 m*M* Tris acetate

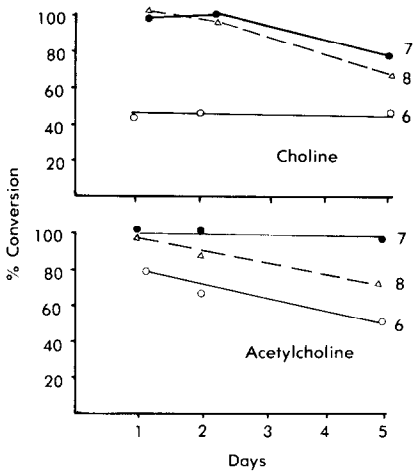

Fig. 2. Stability of choline oxidase (top panel) and cholinesterase (bottom panel) as a function of days of storage at room temperature at pH 6, 7 or 8.

buffer (pH 6, 7 or 8), containing 1 m*M* tetramethylammonium chloride, and then stored at room temperature. At intervals, the cartridges were tested for conversion of ACh to Ch and Ch to peroxide, as described above, at the same pH in which the cartridge had been stored. One day after loading, the % conversion reflected the pH optima of the enzymes, *i.e.* greater at pH 7 and 8 than at pH 6 (Fig. 2). The pH optimum for purified choline oxidase is about 7.5[10], but may change slightly on immobilization. The pH that was best for maintenance of activity during storage appears to be about pH 7. By 5 days after loading, conversion of ACh to Ch was still nearly quantitative, and conversion of Ch to peroxide was greater than 75%.

DISCUSSION

The adsorption of a peroxide-generating enzyme on an ion-exchange cartridge offers an elegant solution to the problem of measurement of a compound with no obvious detectable functional groups. The enzymes can be fixed simply by injection into the mobile phase stream, and washed off or replenished at will. The adsorbed enzyme is surprisingly stable; the cartridge needs replenishment with fresh enzyme only once every 5 to 10 days, even when left at room temperature.

The technique can obviously be extended to other substances for which oxidases are available, such as xanthines[6], sterols, monoamines, polyamines, sugars, and even the stereospecific detection of D- and L-amino acids. Many other types of compounds for which oxidases are not available can be analyzed by use of enzymes when products are readily detectable or when suitably rapid chromogenic or fluorogenic reagents are available for the products of the enzymatic reaction. For example, a phospho-sugar would produce a sugar and inorganic phosphate on exposure to alkaline phosphatase; the phosphate is readily detectable by formation with molybdate of a complex absorbing at 340 nm. Similarly, N-acyl amino acids could be cleaved by an acylase to give a free amino acid, which would react with *o*-phthalaldehyde to give a fluorescent product.

A limitation of the enzyme-loaded cartridge is that there must not be retention of the analyte or product on the cartridge. Thus, anions such as oxaloacetate or phospholipids would probably give broad peaks when passed over an enzyme, bound to an anion exchanger. However, if cation exchangers (which would not retain anionic analytes) were used, they might also poorly retain anionic enzymes. Similarly, adsorption to an anion exchanger is not likely to be useful with dehydrogenases, where the cofactor (NADH) might also be adsorbed. Covalent binding has been successfully used for such assays[1–5]. Another problem in the use of enzyme loaded cartridges is that a mobile phase must be found which will allow an analytical separation of the desired compound without elution or damage to the enzyme. Thus, high concentrations of salt or organic solvent must be avoided, as well as extremes of pH and temperature or harsh reagents. High salt concentrations cannot be used with an AX300 column because of elution of the enzyme. The AX300 column is apparently made by adsorption of polyetheleneimine to silica and the crosslinking it with a diepoxy, such as Epon 828 or butanedioldiglycidyl ether[11]. A short column (3–5 cm), similar to the AX300 column, can be prepared from silica, PEI, and a cross-linker in the laboratory[11], but this is not likely to be cost-effective for occasional laboratory use. However, such a column would serve as a good base for modification of silica to give it desired retention characteristics. It seems likely that, if the column were made more hydrophobic, the combination of ion-exchange and hydrophobic-interaction characteristics, would cause the enzyme to be much more strongly adsorbed. Hofstee found[12] that agarose, derivatized with hydrophobic amines, caused an adsorption of active enzymes that could not be reversed with either strong or weak salt solutions. Alternatively, the primary and secondary amines on the AX300 cartridge might provide convenient sites for covalent attachment of enzymes (for example with a carbodiimide) to prevent enzyme elution by a mobile phase containing high concentrations of salts. Highly lipophilic compounds are usually eluted from reversed-phase columns with concentrations of organic solvent which would destroy enzyme activity. However, many enzymes will tolerate high concentrations of non-ionic detergents. Micellar solutions of detergents are able to elute even compounds as lipophilic as aromatic hydrocarbons[13].

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