Use of Carrier-Bound Lipid Substrates in Enzymatic Work

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ABSTRACT

A procedure has been developed for the adsorption of oily or solid substrates of low water solubility onto inert carriers having large specific surface areas. This technique improves the handling characteristics of such substrates, enhances the standardization of enzymes, and facilitates separation of a carrier-bound substrate/product from the reaction mixture.

INTRODUCTION

Interest in the study of enzymatic reactions in accord with the above title originated from our work on the development of what we call the "i-POINT® TTM" (Time-Temperature-Monitor) for controlling the cold chain handling of perishable commodities, such as foods, vaccines, blood products, sensitive pharmaceuticals, photographic films, etc., both deep frozen and refrigerated (1). This simple instrument consists of two plastic pouches, one containing an enzyme and the other the substrate for the enzyme. By opening the seal between the two with gentle pressure, then mixing, the biochemical reaction is started. When a pre-set time-temperature relationship is reached, the TTM will show the end of the reaction by a change of color of a pH indicator. Because of the long periods of storage at various temperatures above and below the freezing point as well as different pH values during the course of the enzymatic reaction, considerable difficulties were encountered to obtain a stable system. Much work has gone into techniques for stabilizing enzymes in high dilution by various means. An equal amount of effort has been spent in efforts to stabilize substrates especially those having low solubility in water. Conventional methods for emulsification of oily substrates or for dispersing particles of solid substrates were unsuccessful especially in systems which are frozen and then thawed; thus, procedures were sought for methods to stabilize enzymatic substrates by the use of certain carriers.

TABLE I

Carriers Used for Enzymatic Substrates

Carrier	Surface area (m^2/g)
Calcium carbonate	< 50
Kaolin	< 50
Talc	< 50
Active carbon	50
Silicates (Mg, Ca, Al, Na)	50-380
Precipitated silica	60-300
Fume silica	50-400-800

TABLE II

Enzymatic Substrates and Enzymes

Substrate	Enzyme
Tricaproin	Pancreatic lipase
Tripropionin	Pancreatic lipase
2-napthylacetate	*Candida* lipase
Cholesterol	Cholesterol oxidase
Cholesterol acetate	Cholesterol esterase
Cortisone	Cortisone dehydrogenase
Androsterone	β-Hydroxy dehydrogenase
Mandelic acid	Mandelic acid dehydrogenase

EXPERIMENTAL PROCEDURES

The experimental procedures as well as techniques of combining substrates of low solubility in water with carriers are described elsewhere (2). However, the methods used are briefly outlined below.

Carriers

A number of different carriers having varying surface area (m^2/g) were tested. Of the materials used, a number of silica preparations and silicates as outlined in Table I were found to be most preferable. Active carbon was also applicable in certain systems when it did not interfere with cofactors. Many of the silica or silicate products are marketed under trade names like Manosil®, Cab-O-Sil®, Fluosil®, Aerosil®, etc., and are well defined as to particle size and surface area per gram.

Substrates

A large number of enzymatic substrates which are difficultly soluble in water may be used. Some of the substrates used in this study are outlined in Table II together with their corresponding enzymes. These substrates and the enzymes were obtained from the Sigma Chemical Company, Worthington Biochemicals, Merck AG and Fluka AG. The trend of the results with all these substrates was very similar except that one had to work out the combination and proportion of substrate for the specific carrier used.

Substrates were adsorbed on carriers by using organic solvents and carefully evaporating off the solvents in a conventional rotary flask. The resulting material was then carefully ground and sieved to obtain definite particle sizes. A particle size of 74 microns was the preferred material in many experiments. Different proportions of substrate to carrier had to be investigated so as to obtain uniform layering, easy handling as a powder, and a good enzymatic reaction. The surface area or pore size of the carrier was

TABLE III

Effect of Varying Amounts of Tricaproin-Carrier on Enzyme Activity[a]

Substrate TC (tricaproin) (%)	Carrier Fluosil® (%)	Time for hydrolysis (min)
100	0	32
20	80	13
40	60	6.5
50	50	3.5
60	40	3.5

[a]Final substrate concentration in reaction mixture: 25 mM. Enzyme concentration; 0.2 units/ml.

TABLE IV

Pancreatic Lipase Activity on Tricaproin

Enzyme concn. (μg/ml)	Time (min) to form unit amount of acid	
	Emulsion	With carrier (powder)
3	7.5	< 1
1	15	1.3
0.3	37	4.5
0.1	140	11.5
0.03	380	32

also considered in order to fit the size of the enzyme molecule.

Enzymatic Assay Technique

Depending upon the nature of the enzyme reaction, different methods were used for the determination of enzymatic activity. Lipase or esterase activity was measured by determining the amount of acid produced in a given time at constant pH and temperature (3). Dehydrogenase or reductase activity was determined spectrophotometrically at 340 nm for the amount of cofactor NADH formed or used up during the course of the enzyme reaction. Cholesterol oxidase activity was determined by following the rate of disappearance of cholesterol and subsequent formation of cholestenone using extraction and gas chromatographic analysis.

RESULTS

Studies of enzymatic reactions of substrates adsorbed on a carrier such as fume silica (Fluosil®) with a surface area of 200 m^2/g showed a reaction pattern similar to tricaproin. With substrate-carrier combinations having different proportions of substrate to carrier, tricaproin and Fluosil® carrier gave the activity levels described in Tables III and IV. Whereas tricaproin used as an emulsion in the conventional manner gave a reaction time of 32 min to produce a unit amount of acid, the same amount of enzyme reacted almost ten times faster when the proportion of substrate-carrier was 50:50 and 60:40. Lowering this proportion with respect to concentration of substrate resulted in decreased enzymatic activity although it was still considerably higher than the control without carrier. The final concentration of tricaproin was 25 mM in all cases. Thus, a minimum of 50% substrate and 50% carrier with regard to tricaproin and the enzyme concerned gave optimal reaction conditions.

In order to study the effect of particle size of the tricaproin-Fluosil® carrier (50:50 proportion), preparations having different particle sizes were tested. The results in Table V show that enzymatic activity was unchanged with the four different particle sizes of substrate carrier combination tested, 53, 74, 105, and 150 microns.

Different carriers having different surface areas and proportions of tricaproin to carrier were tested. Higher reaction rates were obtained with carriers having larger specific surface areas. It was obvious that one could not layer a sufficient quantity of oily or water-insoluble substrates on carriers of low specific area. With tricaproin, best results were obtained with material having areas between 60-200 m^2/g.

TABLE V

Effect of Particle Size of Tricaproin-Carrier Fluosil® (50:50) on Enzyme Activity[a]

Particle size (microns)	Time for hydrolysis (min)
53	130
74	135
105	135
150	135

[a]Substrate concentration in reaction mixture: 25 mM. Enzyme concentration: 0.015 units/ml.

DISCUSSION

Much work has been done on enzymatic or microbiological interconversion of substrates particularly in the case of water-soluble compounds, but difficulties have been experienced when using substrates of little or no solubility in water. Many of these difficulties could be solved by the discovery that such latter substrates can be adsorbed onto carriers and distributed evenly as well-defined particles in the reaction system. One may thus eliminate unstable emulsions, surface active agents, or antifoam agents and thereby create a more physiological condition in carrying out the reactions. Liquid substrates and waxes can be handled with convenience as powdery material. In addition, a simple separation method will remove soluble products from unreacted substrate. In cases where the product is insoluble, both product and unreacted substrate bound to the carrier may be separated from the enzyme solution.

In the course of these experiments using carrier-bound substrates, it was discovered that the rate of the enzymatic reaction is increased by a factor of nearly ten-fold. Still larger increases can be demonstrated with lower enzyme concentrations. This would suggest a better utilization of the carrier-bound substrate which could be of some benefit in process techniques. This also provides a basis for a better understanding of the kinetics of enzyme reactions with such substrates, leading perhaps to studies concerned with membrane-bound lipids.

A number of advantages are thus gained by using carrier-bound substrates in contrast to conventional emulsions of oily substrates:

1. Liquid substrates can be used in powder form and dispersed in an aqueous medium.
2. No surface active agent is needed.
3. Stable suspensions are obtained with definite particle sizes. An aqueous suspension of tricaproin, for example, on the carrier Fluosil® could be sterilized in an autoclave at 120 C without loss of the substrate from the carrier.
4. Reproducible enzymatic reactions are obtained.
5. Easy handling and separability from reaction mixtures are useful in industrial processes.
6. High rates of enzymatic activity which enables the detection of lower concentration of enzymes.

On the basis of the above, a number of useful applications of the carrier-bound substrates can be visualized. Because of the increase in enzymatic activity (specific activity), it becomes possible to detect low enzyme concentrations. This may be particularly useful in diagnostic work, e.g., in blood or organ analysis where organ-specific enzymes can be readily demonstrated and quantitated. We are moving into an age, at least in the developed countries, where the effects of nutritional imbalance or faulty nutrition are on the increase rather than the problems of under-nutrition. Thus, a number of carrier-bound substrates may offer the possibility for early detection of nutrition-related tissue damage or enzyme defects. Also, the carrier-bound substrates may find use in the standardization of enzymes and in processes where enzymes or microorganisms are used to convert one product into another.

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