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Kinetic Studies and Analytical Application of Cholesterol Oxidase and Peroxidase Immobilized to Synthetic Polymer

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Abstract

A method for individual and simultaneous covalent immobilization of cholesterol oxidase and peroxidase to copolymer of acrylonitrile with acrylamide is described. The effect of immobilization on the catalytic properties of the covalently bound enzymes was studied. The immobilized enzymes showed no change in pH optima and an increase in temperature optima, activation energy, and K_m, compared to data received from experiments with soluble enzymes. A small glass column packed with immobilized multienzyme complex was used to develop a method for manual determination of cholesterol in foodstuffs (e.g., in mayonnaise "Olinease"). The method was characterized by high analytical precision (coefficient of variation = 2.67%). The results show high correlation with those obtained by the Kageyama method ($r = 0.986$). The method is economical (the enzyme-carrier conjugate may be used more than 300 times), precise, easy to perform, and less time-consuming than the manual methods utilizing soluble enzymes. The established manual method can be proposed for cholesterol determination in foodstuffs.

Index Entries: Immobilization; simultaneous immobilization; cholesterol measurement; cholesterol oxidase; peroxidase; copolymer of acrylonitrile with acrylamide.

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Introduction

Enzymatic cholesterol measurement is one of the most expensive tests conducted in biochemical laboratories. The use of immobilized enzymes, which are reusable and relatively stable, appears to be the best approach. The immobilization of cholesterol oxidase (CHOD) and peroxidase (POD) to different carriers is of particular interest because of its application in determination of cholesterol in biological fluids and foodstuffs. More successful in the aspect of stability and reproducibility is the analysis using covalent enzyme immobilization *(1)*. Different carriers and immobilization techniques, by means of chemical modification of amino acid residues of the enzymes, are used *(1–5)*. A method for covalent binding of enzymes to cellulose carriers has been described previously by Krysteva et al. *(6)*. This method includes activation of cellulose matrix using urea and formaldehyde. The obtained derivatives contain active *N*-hydroxymethyl groups, which could bind the amino acid residues of proteins. The method was applied successfully in the immobilization of some enzymes *(7,8)* and in the preparation of multienzyme polysaccharide membranes for biosensors *(9)*. It was also successfully applied for immobilization of enzymes to active synthetic membranes containing copolymer of acrylonitrile with acrylamide *(10)*. To our knowledge, the immobilization of CHOD and peroxidase on organic carrier has not been described to date, and the two immobilized enzymes have not been used for determination of cholesterol in foodstuffs.

The aims of the present work were to immobilize individually and simultaneously CHOD and peroxidase to copolymer of acrylonitrile with acrylamide, to study the catalytic properties of the produced biocatalyst, and to evaluate the possibility of its use for manual determination of cholesterol in foodstuffs.

Materials and Methods

Chemicals and Reagents

Cholesterol oxidase (EC 1.1.3.6) from *Nocardia erythropolis* was supplied from E. Merck (Darmstadt, Germany). Peroxidase (EC 1.11.1.7) from horseradish was purchased from Reanal (Hungary). Catalase (EC 1.11.1.7) from *Aspergillus niger* was obtained from Biovette (Peshtera, Bulgaria). The enzymes were used without further purification. Cholesterol was purchased from Fluka (Switzerland). 4-Aminophenazone, phenol, formaldehyde, acetylacetone, and Triton X-100 (octylphenol-polyethyleneglycol ether) were obtained from Merck. Copolymer of acrylonitrile with 15% acrylamide was from the Department of Plastics, UCTM (Sofia, Bulgaria). All other chemicals used were available products of high purity.

The color Trinder reagent (2 m*M* 4-aminophenazone and 14 m*M* phenol in 0.1 *M* phosphate buffer, pH 7.0) was prepared following a method described elsewhere *(11)*. This reagent consisted of Triton X-100 (0.05% [v/v])

as a surfactant. Cholesterol standard (15.5 m*M*) was prepared by solubilizing 600 mg of cholesterol in 100 mL of 2-propanol. Working standard solutions in the concentration range of 1.5–15.5 m*M* were prepared by diluting the stock solution with 2-propanol.

The test for the analysis of cholesterol in foodstuffs utilizing the method of Kageyama *(12)* contained the following components.

1. Reagent 1: 0.9 *M* ammonium phosphate buffer (pH 7.0) consisting of 2.6 *M* methanol and 2200 U/mL of catalase.
2. Reagent 2: 0.05 *M* acetylacetone, 0.09 *M* sodium dodecyl sulfate, and 0.3 *M* methanol.
3. Reagent 3: 1.25 U/mL suspension of cholesterol oxidase, in 3 *M* NaCl solution.
4. Reagent 4 (solution in the assay): three parts of reagent 1 and two parts of reagent 2 mixed and left at room temperature for 1 h.

Preparation of Granulated Carrier

Forty grams of copolymer of acrylonitrile with acrylamide were purified twice by dissolving in dimethyl formamide (DMF) followed by water precipitation and drying at 37°C for 24 h. The purified copolymer was dissolved in DMF to a concentration of 8%, and was used further for granulating. This was performed by dropping the polymer solution into water: methanol (7:3 [v/v]) containing 8% NaCl. The obtained granules had good porosity and sphericity ($d < 1$ mm). The stability of the granules was achieved by dispersing them in distilled water for 30 min followed by heating at 85°C for 45 min. The granules were further dried and heated at 70°C for 3 h.

Immobilization of CHOD and Peroxidase on Granulated Carrier

The granulated copolymer of acrylonitrile with acrylamide (in which the acrylamide units amount to 15%) was used as a carrier for the immobilization of the enzymes. The copolymer was activated by 12.5% formaldehyde (dissolved in 0.1 *M* phosphate buffer, pH 7.5) and stirred for 4 h at 45°C in a closed vessel *(10)*. The granules were than washed thoroughly with distilled water until there was no formaldehyde in the rinsing waters. The individual immobilization of enzymes was followed by an immediate treatment of the activated carrier (200 mg) with suspension of CHOD (0.2 mg/mL) in 0.1 *M* phosphate buffer (pH 6.8) or with suspension of peroxidase (6.2 mg/mL) in 0.1 *M* citrate phosphate buffer (pH 4.0) for 20 h at 4°C under careful magnetic stirring. The simultaneous immobilization of the two enzymes was performed as follows: To 200 mg of activated carrier were added 0.2 mL of CHOD (1.25 U/mL) and 4 mL of 0.1 *M* phosphate buffer (pH 6.8) with continuous mixing at 4°C for 2 h. Next, 26 mg of peroxidase was added to the mixture and mixing continued for 20 h. The obtained carrier-enzyme conjugates were washed consecutively with

0.1 *M* phosphate buffer (pH 6.8), distilled water, 0.1 *M* NaCl, and again with water until a complete elimination of the absorbance at 280 nm in the rinsing waters was observed. The immobilized enzymes were used immediately or stored in a moist state at 4°C. The amount of bound protein was measured using a modified Lowry's method *(13)*.

Enzyme Activity Assay

The activities of CHOD and peroxidase were determined using a Trinder reagent *(14)*. The increase in the absorbance at 500 nm was measured using a Spekol 11 spectrophotometer (Carl Zeiss, Jena, Germany). One unit of CHOD activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μ*M* cholesterol/min at 37°C and at pH 7.5 to 4-cholesten-3-one in a peroxidase-phenol-4-aminoantipyrine system. One unit of peroxidase activity was defined as the amount of enzyme that caused the decomposition of 1 μ*M* hydrogen peroxide/min at 25°C and pH 7.0.

Determination of pH Optima

For the pH range of 5.0–8.5, the residual activities of soluble and immobilized enzymes were determined in 0.1 m*M* phosphate buffer at 25°C.

Determination of Temperature Optima

For the temperature range of 25–60°C, the residual activities of soluble and immobilized enzymes were determined in 0.1 m*M* phosphate buffer at pH 7.5 for CHOD and pH 6.0 for peroxidase.

Evaluation of Michaelis Constants

The Michaelis constants, K_m, of soluble and immobilized enzymes were calculated at increasing substrate concentrations in 0.1 m*M* phosphate buffer at the optimal pH (2.6–15.5 m*M* cholesterol for CHOD and 0.05–1.5 m*M* hydrogen peroxide for peroxidase). The K_m values were calculated using a computer program.

Estimation of Activation Energy

The activation energy, E_a, was estimated by using the Arrhenius equation for the temperature range of 20–50°C. The value of E_a was determined from the slope of the straight line that resulted when the logarithm of the reaction constant, k, was plotted vs $1/T$ *(15)*.

Determination of Cholesterol in Mayonnaise

Preparation of Sample *(12)*

One gram of mayonnaise and 1 g of sea sand were weighed in a 50-mL round-bottomed flask. Ten milliliters of a freshly prepared methanolic potassium hydroxide solution (1 *M*) was added, and the mixture

was heated under a reflux condenser for 20 min while stirring. The supernatant solution was transferred into a 25-mL volumetric flask by using a pipet. The residue was boiled twice, each time with a portion of 6 mL of 2-propanol under a reflux condenser for 5 min. The solutions were collected in the volumetric flask and were left to cool. The contents of the volumetric flask were diluted with 2-propanol up to the mark and mixed. The turbid solutions were filtered through a fluted filter paper. The filtrate was used for the assay of cholesterol at 37°C.

Performance of Assay

Using Kageyama Method

The assay was performed according to ref. *12*. Five milliliters of solution in the assay (reagent 4) and 0.4 mL of sample solution containing cholesterol were pipeted into a test tube. Next, 0.2 mL of CHOD (reagent 3) was added to 2.5 mL of this sample. The contents of the test tube were mixed thoroughly and incubated in a water bath at 37°C for 60 min. The concentration of the cholesterol was measured by the increase in absorbance at 405 nm using a calibration curve.

Using a Column Packed with Simultaneous Immobilized CHOD and Peroxidase

Into a test tube, 0.3 mL of Trinder's reagent and 0.2 mL of sample solution were pipeted. The contents of the test tube were mixed thoroughly and put in a small glass column (30 mm in length and 10 mm in inner diameter) *(16)* loaded with simultaneously immobilized CHOD and peroxidase (about 0.450 g). A constant-temperature circulating bath was used to maintain the reaction temperature at 37°C. After 20 min of incubation, 4 mL of distilled water was added and the liquid was eluted by vacuum pump. The resulting absorbance of the dyed product was measured through a glass cuvet with a 1-cm light path at 500 nm. The cholesterol content (millimolar) was calculated using a calibration curve. After washing with 5 mL of 0.1 *M* phosphate buffer, pH 7.0, the column was ready for storage or further assays.

Statistical Methods

The results were evaluated by methods of descriptive statistics and linear regression to assess correlation *(17)*. Simple regression analysis of measured cholesterol concentration was accomplished by using the computer program State VieW 512+™ from Brain Power, Calabasas, CA.

Results and Discussion

Cholesterol was oxidized enzymatically to 4-cholesten-3-one by means of immobilized CHOD with simultaneous formation of hydrogen peroxide. The hydrogen peroxide reacted with phenol and 4-aminophenazone (Trinder reagent) in the presence of peroxidase to form an *o*-quinoneimine,

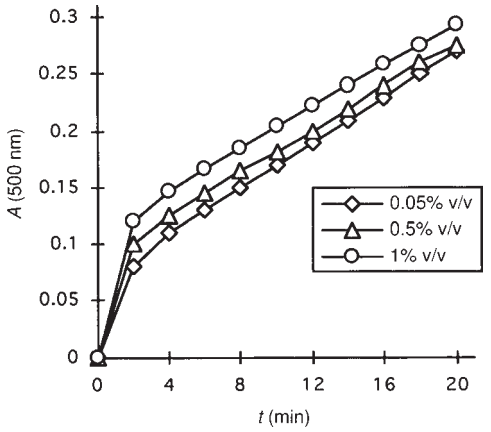

Fig. 1. Effect of surfactant concentration of Triton X-100 on reaction rate. The cholesterol concentration was 13 m*M*.

a colorful oxidation product. The intensity of the color was proportional to the degree of cholesterol oxidation. An increase in absorbance was measured at 500 nm. When the Kageyama method was used, hydrogen peroxide reacted with methanol, generating formaldehyde, which subsequently reacted with ammonia and acetylacetone to form a stable lutidine derivative, which was measured colorimetrically at 405 nm *(12)*.

Cholesterol standards contain a surfactant Triton X-100, and sodium azide (0.1% [v/v]) as a preservative. Triton X-100 (a nonionic surfactant) is an effective lipid-solubilizing agent. The use of a solubilizing surfactant for preparing the cholesterol standards or solutions in the range of concentration from 0.05 to 0.1% (v/v) did not essentially affect the reaction rate (Fig. 1).

The CHOD and peroxidase were bound individually and simultaneously to synthetic copolymer of acrylonitrile with acrylamide. The proposed method of enzyme immobilization probably involved the reactions in Fig. 2. The copolymer was modified with formaldehyde. In the presence of formaldehyde, the amide groups of the copolymer converted to highly reactive *N*-hydroxymethyl groups capable of forming stable covalent bonds with enzymes *(10)*. The binding of proteins to *N*-hydroxymethyl groups of the carrier was dependent on the pH of the reaction mixture. Study of the coupling reaction with both enzymes showed that the highest enzyme activity was achieved at a pH range of 6.8–7.8 for the CHOD and at a lower pH (4.0–4.2) for the peroxidase. The condensation process in the acid pH probably included asparagine (Asn) and glutamine (Gln) *(6)*. The simultaneous immobilization of CHOD and POD was performed at pH 6.8, where the enzymes showed good stability.

The immobilized enzymes were characterized with respect to their pH and temperature optima, activation energy, and K_m, and these values were then compared with those of the free enzymes (Table 1). Table 1 indicates

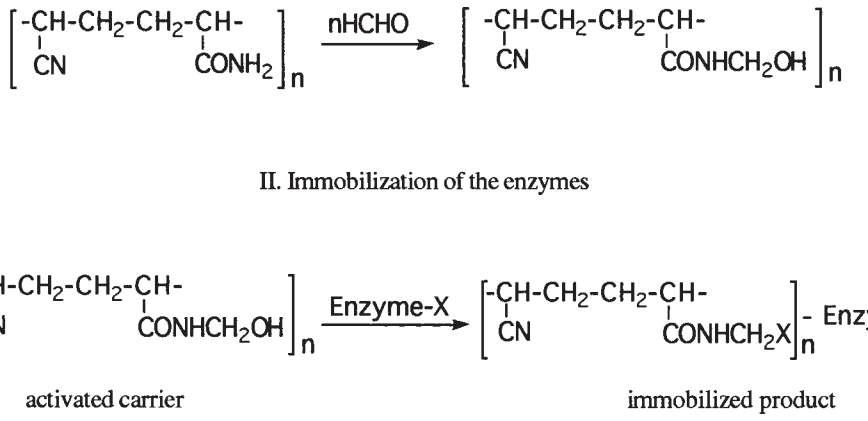

Fig. 2. Scheme of the covalent coupling of CHOD and peroxidase to activated copolymer of acrylonitrile with acrylamide.

that the immobilized CHOD and peroxidase showed no change in pH optima. The immobilized enzymes also showed relatively good activity. The highest activity of immobilized CHOD and peroxidase was obtained at pH values of 7.5 and 6.0, respectively. The temperature optima of the immobilized enzymes tended to increase (up to 10°C for the immobilized CHOD and up to 5°C for the immobilized peroxidase).

As seen in Table 1, the activation energies of the immobilized enzymes were higher than those of the free enzymes (46.8 kJ/mol for the immobilized CHOD vs 31.0 kJ/mol for the free enzyme and 38.2 kJ/mol for the immobilized peroxidase vs 16.6 kJ/mol for the free enzyme). This could suggest that the immobilization procedure caused some conformational changes in the covalently bounded enzyme molecules. The increase in K_m of the immobilized CHOD and peroxidase might be caused by the altered structure of the immobilized enzymes or by diffusional control of the enzyme-substrate interaction.

The present new method for covalent binding of CHOD and POD to copolymer of acrylonitrile with acrylamide tended to show adequate values of the bound protein (Table 1) when compared to methods utilizing other carriers (7.3–9.0 mg/g of dry carrier of individually immobilized enzymes vs 0.7–13 mg/g of dry carrier) *(1,11)*.

The simultaneously immobilized CHOD and POD were used repeatedly to assess their stability in time. The obtained conjugates exhibited good stability. After 3 mo of storage at 4°C, the activity of immobilized enzymes was not practically decreased. When not in use, they were stored at 4°C in 0.1 *M* phosphate buffer (pH 7.0).

The column packed with simultaneously immobilized CHOD and POD (multienzyme column) was successfully used for manual analysis of

Table 1
Catalytic Properties of Immobilized Enzymes[a]

Enzyme	Amount of bound protein (mg/g dry carrier)	pH optimum	Temperature optimum (°C)	Specific activity (U/mg)	Relative activity (%)	E_a (kJ/mol)	K_m (m*M*)
CHOD	7.3	7.5 (7.5)	55 (45)	0.79 (1.25)	63.2	46.8 (31.0)	15.40 (5.71)
Peroxidase	9.0	6.0 (6.0)	35 (30)	1.20 (2.50)	48.0	38.2 (16.6)	3.86 (1.64)
Simultaneously	3.6	—	—	0.50	40.0	—	—
immobilized CHOD				0.80	32.0		
and POD							

[a]Numbers in parentheses refer to the free enzymes.

Table 2
Within-Run Imprecision of Cholesterol Measurements (n = 20)
Using Kageyama Method and Multienzyme Column Method

Sample	Kageyama method	Multienzyme CHOD/POD method
$\overline{X}$ (m*M*)[a]	2.80	3.00
S (m*M*)[b]	0.10	0.08
CV (%)	3.57	2.67

[a]Mean values.
[b]Standard deviation.

cholesterol in foodstuffs (e.g., in mayonnaise "Olinease"). The use of simultaneously immobilized CHOD and POD in this form is attractive mainly owing to the stability of the product, which makes it possible to utilize the same conjugate more than 300 times and is therefore more economical. The multienzyme column reactor showed good operational stability within 2 mo of use. The initial activity of the simultaneously immobilized enzymes decreased about 20% after being reused for 60 d, which allowed good reproducibility of measurements. A linear response was obtained even when the activity of immobilized enzymes decreased to 20% of the initial values. The relation among various cholesterol concentrations and the absorbance (linearity test) was checked with dilution of the stock standard solution. No relevant deviation in the range investigated (1.5–15.5 m*M*) was observed by the proposed CHOD/POD method. The calibration curve for the assay of the cholesterol was linear up to a concentration of 15.0 m*M*. This is in the range of concentration that is generally encountered in sera and foodstuffs. The range of linearity was slightly reduced (up to 13 m*M*) when the Kageyama method was used.

The multienzyme column allowed accurate estimation of cholesterol. The within-run imprecision of the cholesterol determination *(17)* was evaluated by calculation of the variation coefficient of variation (CV) (2.67%) after measuring 20 consecutive samples of mayonnaise (mean values of 3 m*M* cholesterol) vs CV = 3.57% with the Kageyama method (Table 2). The established CV was in good agreement with those found in the literature, which range from 2 to 6% *(18)*.

To estimate the precision of the proposed manual method, we performed simultaneous measurements of cholesterol by the Kageyama method, which utilized soluble enzymes. The Kageyama method is widely applied for the analysis of cholesterol in foodstuffs *(12)*. Data obtained by both methods were compared using simple regression analysis (Fig. 3). The obtained regression equation was $y = 1.116\ x + 0.217$. Our manual method was in good correlation with the Kageyama method ($r = 0.986$). Statistical calculation data confirmed the precision of the proposed method.

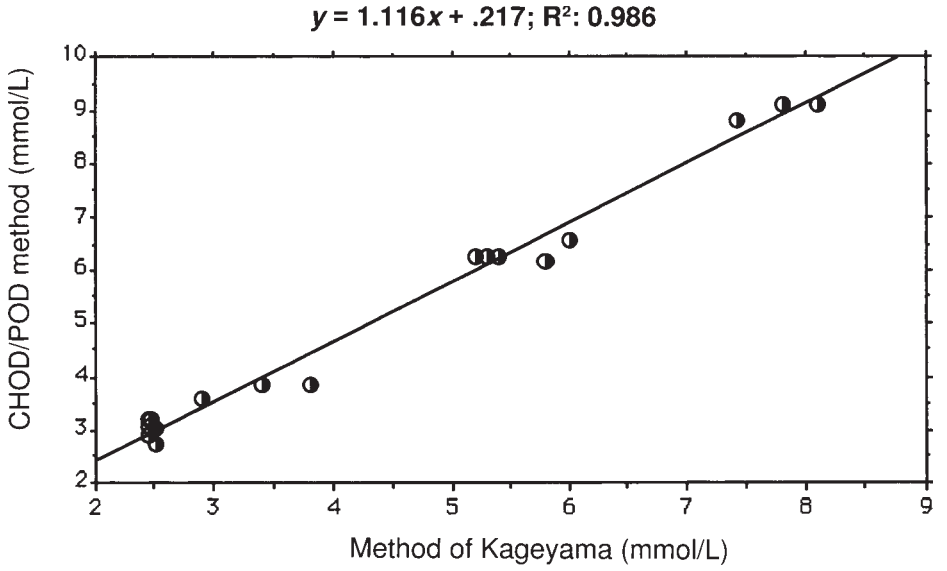

Fig. 3. Correlation between the cholesterol measurements by the simultaneously immobilized CHOD and peroxidase (CHOD-POD method) and by the Kageyama method.

Conclusion

The proposed method of covalently binding CHOD and peroxidase to synthetic polymer of acrylonitrile with acrylamide possesses several advantages. The obtained copolymer carrier is inexpensive and easy to cast in granulated form; activation of carrier completed in a one-step reaction with formaldehyde and the binding of enzymes is simple to carry out; the received multienzyme system shows a high operative stability and capability for cholesterol determination; and the method is specific, precise, and characterizes with good reproducibility. Because of these advantages, the present manual multienzyme column method appears quite promising and attractive for analysis of cholesterol in foodstuffs such as mayonnaise, noodles, and milk.

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