ENZYMATIC SENSOR FOR CHITOSAN DETERMINATION

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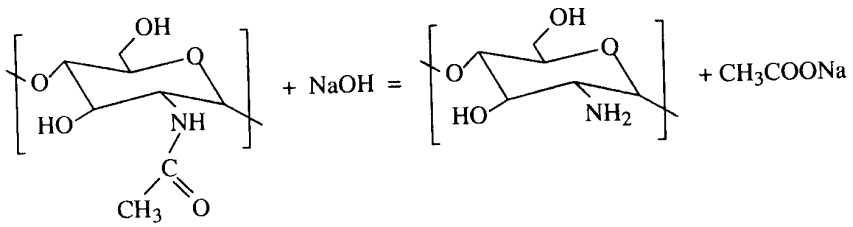
Abstract

A simple and sensitive sensor for chitosan determination based on its activatory effect on the catalytic activity of horseradish peroxidase was proposed. The reaction of o-dianisidine oxidation by hydrogen peroxide was used as an indicator. The enzymatic reaction rate was monitored spectrophotometrically (λ = 440 nm) by an increase of absorbance in a solution due to the formation of the colored products. The optimum conditions of the indicator reaction were studied. It was established that the activatory effect of chitosan on the catalytic activity of horseradish peroxidase depended on nature, ionic strength and pH of the buffer solution. The maximum influence of chitosan on the indicator reaction rate was observed in 0.05 M potassium hydrogenphthalate (pH 5.0) and 0.1 phosphate (pH 6.0) buffer solution. The optimum concentrations of o-dianisidine and hydrogen peroxide are 0.05 and 0,25 mM, respectively. The applicable concentration range of chitosan is 0.0005 – 0.008 w/v % (40 – 670 nM). The relative standard deviation at the lower limit of its concentration range is 6 % (n=5).

Introduction

The problem of chitin utilization which is a basic component of Crustacea shells of crabs and shrimps has a great importance (1-3). The reprocessing of chitin with chitosan formation by the method of alkaline hydrolysis is widly used. The reaction of chitin deacetilation is presented in scheme 1:

chitin chitosan



Scheme 1

Chitosan is characterized by very high biological activity and it is widely used in medicine, pharmacology, cosmetic and food industry (4). At the same time there are no simple and express methods for chitosan determination in the technological process of chitin deacetilation. In this work we present a new sensitive procedure for the determination of chitosan based on its activatory effect on the catalytic activity of horseradish peroxidase.

Methodology

Reagents

Horseradish peroxidase (HRP) (EC 1.11.1.7, RZ = 2.2) ("Sigma"; USA) was used. Aqueous enzyme solutions were obtained by dissolving the commercial enzyme preparation in 0.05 M sodium borate buffer (pH 7.0) which contained 20 vol. % 0.05 M sodium nitrate to maintain constant ionic strength. The concentration of the peroxidase solution was measured spectrophotometrically (λ = 403 nm, *l* = 1 cm, and $\varepsilon = 9.4 \cdot 10^{4}$ l mol $^{-1}$ cm^{-1}). The enzyme solutions were stored in a refrigerator at 4 ^{0}C.

Phosphate buffer solutions (pH 5.2 – 7.5) were prepared by mixing ammonium dehydrophosphate and ammonium hydrophosphate. Potassium hydrogen phthalate buffer solutions (pH 4.2 – 6.2) were prepared by mixing solutions of potassium hydroxide and potassium hydrogenphthalate.

o-Dianisidine and hydrogen peroxide reagents were purchased from Reakhim, Moscow. Solutions of o-dianisidine (Reakhim, Moscow) were prepared daily by dissolution of its accurately weighed amounts in ethanol.

Chitosan with molecular weight 120 kDa was prepared from Crab shells chitin (Vladivostok). 1-1000 nM chitosan solutions were prepared by dissolution of its accurately weighed amounts in 5% acidic acid.

All the reagents were of analytical grade. All the solutions were prepared using deionized water (Millipore, France).

Instrumentation and measurement procedure

Absorbance of peroxidase solutions was measured with an UV-2201 spectrophotometer ("Shimadzu", Japan), pH of solutions was measured by pH-millivoltmeter ("Ekonikc-Expert", Russia).

The rate of all the indicator reactions was monitored spectrophotometrically (λ = 440 nm) by an increase of absorbance in a solution due to the formation of the colored products. All experiments were carried out at room temperature. Note that the rate of oxidation of peroxidase substrate changes by up to 2% when the temperature changes by 1^{o}C.

Analytical procedures

a. o-Dianisidine oxidation catalyzed by peroxidase

Indicator reaction was carried out in the following way: the definite volume of buffer solution to make the total volume of the reaction mixture equal to 5 ml, 0.1 ml of the peroxidase solution (5 nM), 0,1 ml of 50 μM ethanolic o-dianisidine, and finally, 0.1 ml of 0.25 mM hydrogen peroxide were sequentially introduced into a glass test tube with a ground-glass stopper. At the moment when hydrogen peroxide was added and the solution was mixed, a stopwatch was started and the absorbance was measured at 15 s intervals for 2 min. The rate of the indicator reaction was characterized by the slope (tg α) of the linear absorbance – time plot.

b. Chitosan determination using the reaction of o-dianisidine oxidation catalyzed by horseradish peroxidase was carried out with help of almost the same procedure which was described above. The only difference is the addition of the essential volume of 0.5% solution of chitosan in its concentration range of 40-670 nM (0.0005 – 0.008 w/v %) to the reaction mixure just after introduction of the enzyme solution.

The extent of chitosan activatory effect on peroxidase catalytic activity was calculated according to the formula:

$$A, \% = 100\ \% \cdot (tg\alpha_{activator} / tg\ \alpha_{control}) - 100\ \% \qquad (1),$$

$tg\alpha_{activator}$ and $tg\ \alpha_{control}$ – the rate of the indicator reaction in the presence and absence of chitosan, respectively.

Results and discussion

Influence of chitosan on the catalytic activity of peroxidase in the reaction of o-dianisidine oxidation

The reaction of *o*-dianisidine oxidation by hydrogen peroxide was used as indicator to control the catalytic activity of peroxidase. The optimum conditions of the reaction were chosen and presented in Table 1.

Table 1: The optimum conditions of the indicator reaction

The reagent	Concentration
HRP	0.1 нМ
o-Dianisidine	0.05 мМ
H_2O_2	0.25 мМ

Chitosan is a polyelectrolyte polymer, and it was supposed that the effect of chitosan on the catalytic activity of peroxidase would depend on the nature, pH and concentration (ionic strength) of the buffer solution in the indicator reaction.

The influence of chitosan on the catalytic activity of peroxidase in phosphate (pH 5.2 – 7.5) and potassium hydrogen phthalate buffer (pH 4.2 – 6.2) solutions was studied (Figures 1-3). It was established that chitosan in its concentration range of 40-670 nM (0.0005 – 0.008 w/v %) was an activator of peroxidase, and its activatory effect was much higher in potassium hydrogen phthalate buffer solution (Figure 1).

The activatory effect of chitosan on the catalytic activity (~ 68 %) of the enzyme was maximum in 0.05 M hydrogen phthalate buffer (pH 6.0). The obtained data are presented in Figures 2-3.

Figure 1: The influence of chitosan concentrations on the catalytic activity of horseradish peroxidase in potassium hydrogenphthalate (1) and phosphate (2) buffer solutions

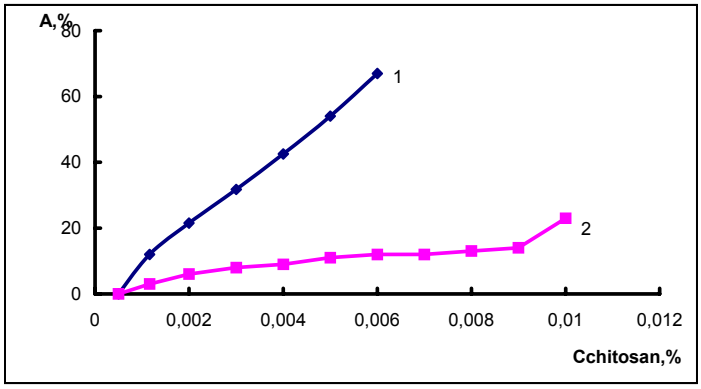


Figure 2: The activatory effect of chitosan on the catalytic activity of horseradish peroxidase in the presence of different concentrations of potassium hydrogenphthalate (pH 6,0) buffer solutions

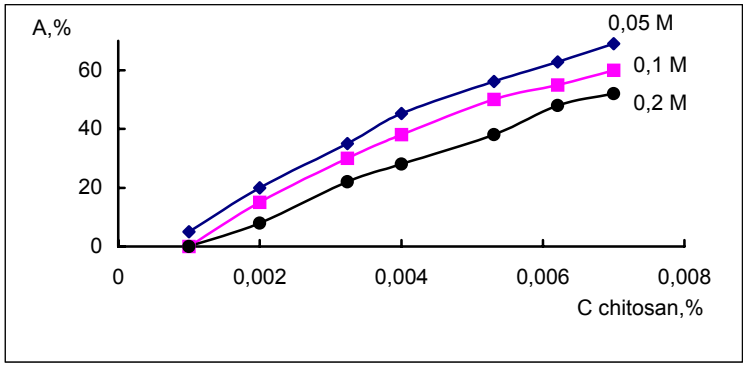
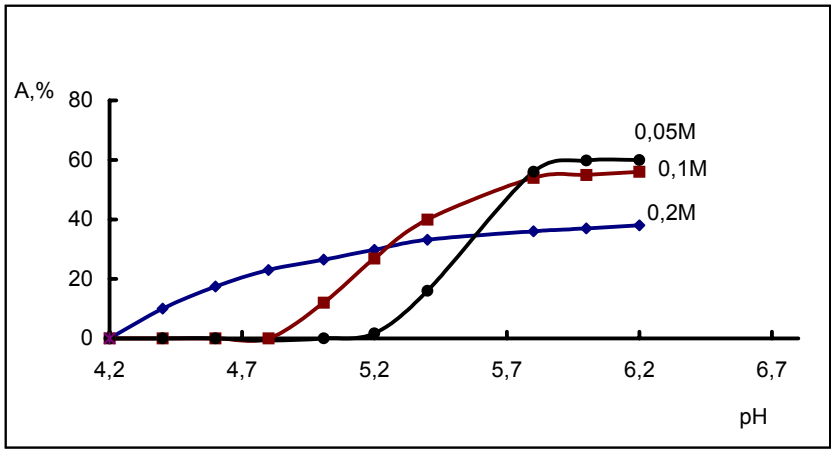


Figure 3: The influence of pH of 0,05 M hydrogen phthalate buffer solution on the activatory effect of 0,004 w/v % chitosan in the reaction of o-dianisidine oxidation catalyzed by peroxidase



The activatory effect of chitosan on the peroxidase catalytic activity may be obviously explained by a formation of a polyelectrolyte complex of the enzyme with polysaccharide which is characterized by much higher catalytic activity than native enzyme. Earlier this effect has been noted by the example of complex formation of chitosan with another enzyme - chitinase (5).

The development of the procedure for the determination of chitosan

The optimum conditions of *o*-dianisidine oxidation (Table 1) in 0.05 M hydrogen phthalate buffer (pH 6.0), where activatory influence of chitosan on the catalytic activity of peroxidase was maximum, were used to develop a procedure for its determination (Table 2).

Table 2: Analytical characteristics of the procedure for chitosan determination

Applicable concentration range of chitosan, w/v %	The calibration equation	RSD, %
0.0005 – 0.008	y=1090x+11	6

where y – the degree of the activatory effect of chitosan (A, %); x –the concentration of chitosan, w/v %.

The possibility of application of the developed procedure for the determination of different contents of chitosan in the presence of 0.5 w/v % chitin has been shown (Table 3).

Table 3: Results of chitosan determination in the presence of 0.5 w/v % chitin (n = 5, P = 0.95)

Chitosan concemtration, w/v %	
Introduce	Found
0.002	0.0021 ± 0.0005
0.004	0.0042 ± 0.0006
0.006	0.0059 ± 0.0003

Conclusions

The influence of chitosan on horseradish peroxidase in the indicator reaction of *o*-dianisidine oxidation was studied. It was established that chitosan in its concentration range of 0.0005 - 0.008 w/v % (or 40 – 670 nM) was an activator of horseradish peroxidase.

It has been stated that the activatory effect of chitosan on horseradish peroxidase depends on nature, ionic strength and pH of the buffer solution. The activatory effect (~ 68 %) of the polymer on the catalytic activity of the enzyme was maximum in 0.05 M hydrogen phthalate buffer (pH 6.0).

The simple and sensitive procedure for chitosan determination in the above mentioned concentration range was developed.

References

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