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Preparation of Cu(II) adsorbed chitosan beads for catalase immobilization

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ABSTRACT

Glutaraldehyde pretreated crosslinked chitosan beads (Ch) were used for Cu(II) adsorption (Cu-Ch). The amount of adsorbed copper was determined using atomic absorption spectroscopy.

Adsorption of the copper onto the chitosan was studied batch adsorption technique at 20 °C. In adsorption experiments a Langmiur type (L) adsorption was found with respect to the Giles classification system. Binding parameters such as initial binding constant (*Ki*), equilibrium constant (*K*), monolayer covarege (*n*), site-size (*u*), maximum fractional occupancy (θ) and free energy of adsorption (ΔG) for the chitosan/copper system were calculated Langmiur linearization method.

Glutaraldehyde pretreated crosslinked chitosan beads (with and without copper) were used in catalase (CAT) immobilization. Various characteristics of immobilized catalase such as, the temperature activity curve, thermal stability, operational stability, and storage stability were evaluated. Among them optimum temperature were found to be 25–35 °C and 35 °C for Cu-Ch-CAT, Ch-CAT, respectively. The apparent *K*m value was 18 mM whereas the *V*max was calculated as 4800 μmol (mg protein min)$^{-1}$ for immobilized catalase on chitosan beads (Ch-CAT) at 35 °C. The apparent *K*m value was 53 mM and the *V*max value was 18450 μmol (mg protein min)$^{-1}$ for immobilized catalase on Cu(II) adsorbed chitosan beads (Cu-Ch-CAT) at 35 °C. Operational, thermal and storage stabilities of the Cu-Ch-CAT were higher than that of Ch-CAT. This study demonstrated that the potential of cross-linked chitosan beads applied to metal sorption and enzyme immobilization.

1. Introduction

Immobilized metal affinity chromatography (IMAC) has been developed as a popular chromatographic tool for the purification of enzymes and proteins. Immobilized metal affinity (IMA) adsorbents provide moderate affinity to macromolecules by covalently coupling chelating compounds on solid supports to entrap metal ions (Gaberc-Porekar & Menart, 2001). Biosorption or sorption to material of biological origin is recognized as an emerging technique for the treatment of wastewater containing heavy metals (Gamage & Shahidi, 2007). Chitosan has been described as a suitable biopolymer for the collection of metal ions since the amino groups and hydroxyl groups on the chitosan chain can act as chelation sites for metal ions (Steenkamp, Keizer, Neomagus, & Krieg 2002). Chitosan, which is a poly-*N*-acetylglucosamine, is a transformed oligosaccharide obtained by the deacetylation of chitin (da Silva & da Silva, 2004; Kartal & Imamura, 2005). Both biopolymers are chemically similar to cellulose, differing only in the R group attached to carbon 2 of the general carbohydrate structure. Thus, *N*-acetyl ($NHCOCH_3$) groups are disposed in chitin, amine (NH_2) groups in chitosan, and hydroxyl (OH) groups in cellulose. Due to the features mentioned, chemical and physical properties of these polymers are different in nature. Chitosan is inexpensive, nontoxic, hydrophilic, biocompatible, and biodegradable. Based on its many favourable characteristics, chitosan have attracted considerable attention in the areas such as biotechnology, biomedicine, food ingredients and cosmetics (Singla & Chawla, 2001). The solubility of chitosan in organic acids allows of gel, membrane and bead fabrication (Çetinus & Öztop, 2003; Krajewska, Leszko, & Zaborska 1990). Gel, membrane and beads forms of chitosan have been used enzyme (protein, cell) immobilization as support materials (Altun & Çetinus, 2007; Çetinus & Öztop, 2003; Chiou & Wu, 2004; Freeman & Dror, 1994; Gaur, Pant, Jain, & Khare, 2006; Krajewska et al., 1990).

The potential use of enzyme and cell immobilized chitosan beads carried out in acidic media raises a general problem. As chitosan is soluble in acidic solutions, the continuous prolonged exposure of chitosan beads, made via counter-ion precipitation, may result in gel softening and bead disintegration. A procedure was used for preparation of chemically crosslinked chitosan beads. This procedure is based on the addition of an acidic chitosan solution to a mixture of diphosphate and glyoxal, resulting in a chemically crosslinked chitosan beads (128 μm diameter). Glyoxal crosslinked chitosan beads were exposed to glutaraldehyde

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solution as a complementary stability curing treatment. As Schiff's base formed between chitosan and glyoxal is essentially reversible, continuous prolonged operation under acidic conditions may result in gradual leakage of glyoxal. Glutaraldehyde irreversible crosslinking via Schiff's base may lead to chitosan beads exhibiting high operational stability (Freeman & Dror, 1994).

Catalase is a haem containing metalloenzyme and regarded as one of the most common enzymes in plant and animal tissues. Catalase consists of four subunits, each subunit has ferriporphyrin as a prosthetic group. Catalase which decomposes hydrogen peroxide to water and oxygen has been used in industry for a long time. Immobilized catalase has useful applications in various industrial fields in the removal of hydrogen peroxide used as oxidizing, bleaching or sterilizing agent and in the analytical field as a component of hydrogen peroxide or glucose biosensor systems (Arıca, Öktem, Öktem, & Tuncel, 1999; Ertaş, Timur, Akyılmaz, & Dinçkaya, 2000; Santoni, Santianni, Manzoni, Zanardi, & Macsini, 1997). With immobilized enzymes, improved stability, reusability, continuous operation, possibility of better control of reactions, high purity and product yields and hence more favorable economic factors can be expected (Kennedy & Melo 1990).

The first aim of this study was to prepare Cu^{2+} (which is called borderline acid for enzyme/protein immobilization system) adsorbed chitosan beads and secondly, to perform enzyme immobilization onto this matrix. Catalase which is frequently used in the food industry was chosen as a model enzyme for immobilization experiment, and the kinetic parameters, operational, thermal and storage stability of immobilized catalase in batch systems were investigated. The present work can be provided quantitative information on the binding characteristic of Cu^{2+} with chitosan. Our final purpose was to determine potential use of copper adsorbed chitosan beads as chromatographic column resin agent in protein purification in IMAC.

2. Materials and methods

2.1. Materials

Catalase (hydrogen peroxide oxidoreductase; EC.1.11.1.6) from bovine liver, glutaraldehyde, glyoxal (trimer; dihydrate), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Chitosan (High molecular mass $M_W \sim 600{,}000$ Da) was obtained from Fluka Chemie AG (Buchs, Germany). Hydrogen peroxide, tetrasodium pyrophosphate, cupper(II) nitrate and all other chemicals were obtained from Merck AG (Darmstdat, Germany).

2.2. Preparation of glutaraldehyde pretreated chitosan beads (Ch)

Chitosan beads (average 128 μm diameter) were prepared as described in a previous publication (Çetinus & Öztop, 2003). Briefly, chitosan (3.0 g) was dissolved in 1% acetic acid (100 mL) and extruded dropwise through a syringe equipped with a hypodermic needle into crosslinking solution (glyoxal hydrate solution 4% (w/v) mixed with an equal volume of 3% (w/v) tetrasodium pyrophosphate solution, pH 8.0). Thereafter, the beads were washed with 200 mL 50 mM phosphate buffer solution, pH 7.0. For reinforcement of chitosan beads, they were incubated in cold 0.05% (w/v) glutaraldehyde solution for 1 h. The brownish reinforced beads were washed several times with 0.05 M cold phosphate buffer (pH 7.0) and dried in air and then under vacuum.

2.3. Adsorption of Cu^{2+} on glutaraldehyde pretreated chitosan beads (Cu-Ch)

A stock solution (1000 mgL^{-1}) of Cu^{2+} ions was prepared using $Cu(NO_3)_2$. Chitosan beads (0.5 g) were mixed with 10 ml of Cu^{2+} solutions (100, 200, 400, 600, 800 mg L^{-1} concentration at pH 7.0) for chelating at 20 °C. The flask was stirred at 20 °C in a rotary shaker for 24 h (sufficient to reach equilibrium). The concentration of Cu^{2+} in the resulting solution was determined using a flame atomic absorption spectrometer (Solar Ati-Unicam 929 model FAAS). The blue-colored beads adsorbed Cu^{2+} were washed thoroughly with distilled water and stored in distilled water for further use.

The amount of adsorbed Cu^{2+} was calculated by using the concentration of Cu^{2+} in the initial solution and in the equilibrium. The degree of adsorption was calculated based on the difference of Cu^{2+} concentration in aqueous solution before and after adsorption. In a batch adsorption system at equilibrium, total solute concentration C_1 (mol L^{-1}) is

$$C_1 = C_B + C \tag{1}$$

where, C_B is the equilibrium concentration of the solute on the adsorbent in mol per liter (bound solute concentration) and C is equilibrium concentration of the solute in the solution in mol L^{-1} (free solute concentration).

2.4. Immobilization of catalase on chitosan beads

Immobilization experiments were conducted for 5 h at 20 °C in a rotary shaker. Chitosan beads (0.5 g Ch and Cu-Ch) were immersed in 10 mL of catalase solution (0.2 mg mL^{-1}) in 50 mM phosphate buffer pH 7.0. After immobilization period, chitosan beads (Ch-CAT and Cu-Ch-CAT) were removed from each solution and washed several times with 50 mM (10 mL) phosphate buffer solution (pH 7.0). Total concentration of catalase in the solutions was determined by the method of Bradford (1976) using a UV-VIS spectrophotometer.

The amount of bound protein (Q) was calculated using the following equation:

$$Q = \frac{(C_o - C_e)}{m} xV \tag{2}$$

where Q is bound protein (mg protein/g chitosan beads), C_o and C_e are the initial and equilibrium protein concentrations in the solution (mg/ml), V is the solution volume (ml), and m is the mass of chitosan beads (g).

2.5. IR spectra of chitosan beads

The FTIR spectra were recorded using an MATTSON 1000 Fourier transform infrared (FTIR) spectrometer by ATI/UNICAM (England) and the sample and KBr were pressed to form a tablet.

2.6. Enzyme activity assay

The activity of catalase was determined spectrophotometrically by direct measurement of decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by the enzyme (Aebi, 1981). Hydrogen peroxide solutions (2.5–20 mM) were used to determine the activity. Catalase immobilized chitosan beads (250 mg Ch-CAT and Cu-Ch-CAT) were mixed with 10 mL of hydrogen peroxide solution in 50 mM phosphate buffer (pH 7.0) at 35 °C. After 5 min, the reaction was terminated by removal of the chitosan beads from the reaction mixture. The absorbance of the reaction mixture was determined and immobilized catalase activity was calculated. The effect of substrate concentration on the activity was tested by using increasing concentrations of hydrogen peroxide, Vmax and Km values of Ch-CAT and Cu-Ch-CAT were determined.

2.7. The temperature profiles and thermal stability of free and immobilized catalase

Activity of temperature profiles was determined at indicated temperatures (25–55 °C) using a 10 mM hydrogen peroxide solution as a substrate in 50 mM phosphate buffer (pH 7.0).

The thermal stability of free and immobilized catalase was ascertained by measuring the activity of the residual enzyme exposed at various temperatures (25–65 °C) in 50 mM phosphate buffer (pH 7.0) for 5 h. Activities of samples were determined under optimum conditions (10 mM hydrogen peroxide solution in 50 mM phosphate buffer at 35 °C, pH 7.0).

2.8. The operational stability of free and immobilized catalase

The retention of the immobilized enzyme activity was tested as described in the activity assays of catalase. After each reaction run, the enzyme immobilized chitosan beads were removed and washed with 50 mM phosphate buffer at pH 7.0 to remove any residual substrate within the chitosan beads. They were then reintroduced into fresh reaction medium and enzyme activities were detected under optimum conditions.

2.9. The storage stability of free and immobilized catalase

The activity of free and immobilized catalase after storage in 50 mM phosphate buffer (pH 7.0) at 5 °C was measured in a batch operation mode under the experimental conditions given above.

The results of temperature, reusability and storage stability of immobilized catalase were presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

2.10. Desorption and regeneration studies of Cu-Ch-CAT

To investigate the regeneration of the Cu^{2+} adsorbed chitosan beads, Cu-Ch-CAT were treated with 0.1 M NaSCN, after then 50 mM EDTA (ethylenediaminetetraacetic acid) solutions, and Cu^{2+} adsorption and catalase immobilization procedure was applied again. This Cu^{2+} adsorption–desorption process was carried out five times.

3. Result and Discussion

Protein-metal affinity has a central role in a wide variety of applications in the processing of non-modified or engineered proteins, including matrix-assisted refolding, protein partners identification, protein characterization, phosphoprotein analysis and fractionation, protein purity assessment, protein-folding evaluation, site-specific protein immobilization (biosensors, functional assay plates and molecular interaction studies), protein detection and quantification, protein extraction (two phase metal affinity partitioning) and IMAC coupled to other chromatographic and non-chromatographic protein separation techniques (Ueda, Gout, & Morganti, 2003).

In this immobilization experiment, chemically cross linked chitosan beads were used as a support material. Glutaraldehyde and glyoxal were used as crosslinking agents. These were used for different purposes. Glyoxal was used for the formation of chitosan beads and glutaraldehyde was used for reinforcement of chitosan beads. Unless chitosan beads are reinforcement with glutaraldehyde, beads structure will be very weak and with insufficient operational stability. Degree of cross linking of chitosan beads is very important for copper ions adsorption. Juang, Wu, and Tseng (2002) investigated chemically modified chitosan beads for sorption and enzyme immobilization. They reported the adsorption of Cu^{2+} onto cross-linked beads decreased with increasing degree of cross-linking. In our study, a lower crosslinking degree (0.05% glutaraldehyde) was maintained to obtain sufficient amount of free amino groups for copper(II) interactions on chitosan beads.

Cu^{2+} adsorption experiments were performed at neutral pH. At low pH values (acidic solution), amine groups in beads are protonated which induces electrostatic repulsion for Cu^{2+}. Therefore, competition exists between protons and Cu^{2+} for adsorption sites and adsorption capacity can be decreased. At higher pH values (basic solution), precipitation of Cu(II) hydroxide occurs simultaneously with the adsorption of Cu^{2+}, hence, one needs to be careful in interpreting the adsorption capacity of the beads (Wan Ngah, Endud, & Mayanar, 2002). A pH of 7.0 was chosen for the adsorption of Cu^{2+} to avoid the formation of Cu(II) hydroxide which will affect the adsorption capacity by the beads.

As a matter of fact, the presence of NH_2 groups on the chitosan backbone improves its potential during the adsorption process, which is higher than that of chitin. A greater interaction with multivalent cations is expected, being especially important for divalent cations (İshii, Minegishi, Lavitpichayawong, & Mitani, 1995). In some investigations the qualitative preference of chitosan for copper cations was noted (Monterio & Airoldi, 1999; Peter 1995). Considering this feature, some study were conducted to understand the mechanism and structures formed upon complex formation, involving the final chitosan-copper interaction (Rinaudo & Domard, 1989) It is proposed in many cases that the amine group on chitosan is essential to establish a defined $[NH_2]/[Cu]$ ratio. On the other hand, the presence of second interaction of copper with the hydroxyl group of carbon 3 is expected. In such a case only a monomer participates in the coordination. However, the other possibility is related to the participation of two distinct monomers of the same, or different, chains embracing the same cation to form a stable complex (Braier & Jishi, 2000; Steenkamp et al., 2002).

3.1. Adsorpsion isotherm of Cu^{2+} on chitosan beads

The value of the bound concentration may be obtained from Eq (1). For a fixed free solute concentration, C_B, is proportional to the chitosan concentration in the binding system; the amount of binding can therefore be conveniently expressed as the binding ratio, r, defined by

$$r = C_B/P \tag{3}$$

Thus with CB in mol per lit and P is mol (moles of monomer units) in per lit, r then represents the average number of molecules of solute bound to each monomer unit at that free solute concentration (molar mass of monomeric units of chitosan was taken as 179 g mol^{-1}).

Plots of the binding ratio (r) against the free concentrations of copper in the solutions (C_d, mmol copper L^{-1}) are shown in Fig. 1. Fig. 1 shows that adsorption of the Cu^{2+} within the chitosan corresponds to type L (Langmiur *type*) adsorption isotherms in the Giles classification system for adsorption of a solute from its solution. In this type of adsorption isotherm, the initial curvature shows that as more sites in the substrate are filled it becomes increasingly difficult for a bombarding solute molecule to find a vacant site available. This implies either that the adsorbed solute molecule is not vertically oriented or that there is no strong competition from the solvent (Giles, Dsilva, & Easton, 1974).

The types of system which give this curve do in fact fulfill these conditions. Therefore they must have one of the following characteristics: (i) the adsorbed molecules most likely are adsorbed flat or (ii) if adsorbed end-on, they suffer little solvent competition; examples of (ii) are (a) systems with highly polar solute and adsor-

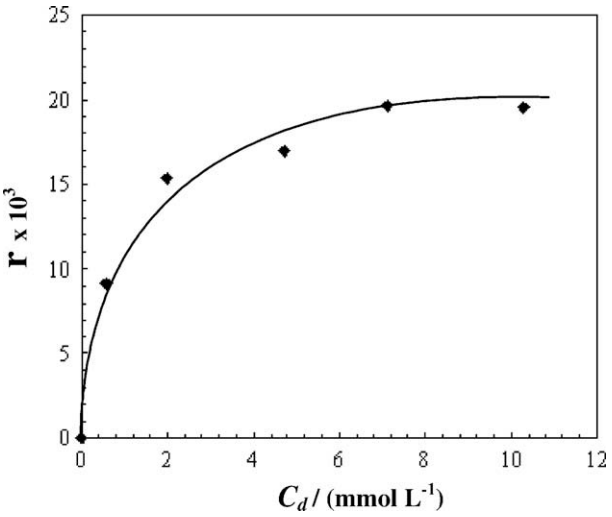


Fig. 1. Binding isotherm of chitosan/copper system. Adsorption study of copper on chitosan beads was performed at 20 °C at several Cu^{2+} concentrations. Binding ratio, *r*; free concentrations of copper in the solutions, C_d.

bent, and a non-polar solvent, and (b) a system in which monofunctional ionic substances with very strong intermolecular attraction, are adsorbed from water by ion–ion attraction. It is possible that in this case (system b) the adsorbed ions may have conglomerated into very large clusters for adsorption to take place (Giles et al., 1974).

The binding data was interpreted on the basis of the uniform site-binding model (u.s.b.). This model based on statistical-thermodynamic terms corresponding to the formation of an ideal localized one-dimensional monolayer of solute on the polymer chains (Molyneux & Vekavakayanondha, 1986). This leads to the hyperbolic (Langmiur) from the binding isotherm, which applies to many polymer/solute binding system.

$$r = \frac{nKC}{1 + KC} \tag{4}$$

where *K* is the binding constant, i.e. the equilibrium constant for the attachment of copper (Cu) onto a site (Ch) by a specific combination of non-covalent forces

$$Cu + Ch = Cu \sim Ch$$

and *n* is the site density i.e. the limiting value of *r* for "monolayer" coverage, which is therefore of density of the sites Ch along the chitosan chain. The reciprocal of n is the site-size, *u*, which may be taken to represent either average number of monomer units occupied by the bound solute molecule, or more generally the average spacing of solute molecules when the chain is saturated. The initial binding constant, *Ki* is the initial slope of the binding isotherm, and therefore average binding strength of a solute molecule by a single monomer unit on an occupied chain. In the u.s.b. model it is equal to the product *Kn*.

There is a wide variety of methods available for testing whether or not the data from a particular binding system fits the hyperbolic of Eq. (4) and for obtaining the 'best' estimates of the parameters *K* and *n*.

Whatever the model for testing and fitting is chosen, the values *K* and *n* obtained should always be checked by plotting the predicted binding isotherm and showing that the experimental data does fit this within the statistically acceptable error limits. If the fit is not satisfactory, the parameters *K* and *n* may be adjusted either until the optimum fit is obtained, or until it is evident that the data cannot in fact be fitted satisfactorily to this model.

To get the best values for the binding parameters from the experimental data, the linearization methods of Eq. (4) have been developed by some researches such as Langmiur (Molyneux, 1984).

When Eq. (4) is rearranged as below:

$$\frac{C}{r} = \frac{1}{nK} + \frac{C}{n} \tag{5}$$

so that here a plot of *C*/*r* vs. *C* should be straight line slope *1*/*n*, ordinate intercept *1*/*nK*.

Binding parameters for the Chitosan/Cu^{2+} system were calculated from the intercepts and slopes of the Langmuir plots.

The derived values of the binding parameters *K* and *n* are listed in Table 1 for copper with chitosan. The final column contains the derived values of the θ, the maximum fractional occupancy attained experimentally, calculated from the definition of fractional occupancy θ

$$\theta = \frac{r}{n} \tag{6}$$

using the value of *r* at the maximum experimental free copper concentration and with the site-density obtained for the u.s.b. model (Molyneux & Vekavakayanondha, 1986).

The values of the binding parameters calculated from the binding isotherm methods are also given in Table 1.

Adsorption free energy of the chitosan/copper binding system was calculated according to the following equation;

$$\Delta G = -RTlnK \tag{7}$$

As already pointed out, physical adsorption is an *exothermic* process. If adsorption is to take place spontaneously, then the free energy must diminish during the process so that ΔG must have a negative value. ΔH must be negative, i.e., the process is exothermic. (Gregg & Sink, 1982). ΔG value of the system is shown in Table 1.

3.2. Catalase Immobilization

Catalase immobilization experiment were performed at pH 7.0 in 50 mM phosphate buffer to prevent copper ion desorption from chitosan beads. Xi and Wu (2004) reported that capability of protein coordination with the copper ion decreased under acidic and basic conditions. In acidic medium, this tendency may be caused by the protonation of electron donor group in amino acid, leading to decreasing coordination ability with metal ion. But when the pH was higher than 7.0, the adsorption capacity was decreased remarkably, it was explained that the phosphate ion in buffer might compete with the protein to coordinate with copper ion. They showed that, the percentage of HPO_4^{2-} and PO_4^{3-} in buffer was increased when the pH value was high. These two kinds of ions possessed a relatively higher coordinating ability compared to $H_2PO_4^-$. Also these ions competed with protein for metal binding.

In catalase immobilization experiments, chitosan beads (Ch) and Cu^{2+} adsorbed chitosan beads (Cu-Ch) were used as support materials. The bound protein amount (Q) was calculated as 0.115 mg /g for Cu-Ch-CAT and 0.074 mg/g for Ch-CAT. Immobilization yields were calculated as 51.7% and 79.3% for Ch- CAT and Cu-Ch-CAT, respectively. Binding of proteins (or peptides) to metal ions is based on interaction between an electron- donating group present on a protein surface and a metal ion presenting one or more accessible coordination sites.

Mechanism of catalase immobilization on glutaraldehyde-pretreated chitosan beads (without Cu^{2+}) can be proposed as Scheme 1.

Table 1
Binding parameters for chitosan/copper system.

Ki/L mol^{-1}	*K*/L mol^{-1}	*n*	*u*	θ	$\triangle G$/kJ mol^{-1}
31.94	1520	0.021	47.62	0.932	−18

Table 2
Kinetic properties of the free and immobilized catalase.

	*K*m (mM)	*V*max (μmol/mg protein min)	Bound protein (mg/g carrier)
Free catalase	35	32000	–
Ch-CAT	18	4800	0.074
Cu-Ch-CAT	53	18450	0.115

In this stage, firstly, free amino groups of chitosan form Schiff's bases with glutaraldehyde. This structure should introduce free aldehyde groups of glutaraldeyhyde onto chitosan beads to facilitate the reaction with enzyme. After ε-amino group of Lys residues on protein surfaces can bind free aldehyde groups of glutaraldehyde on chitosan beads (Altun & Çetinus 2007; Freeman & Dror, 1994; Krajewska et al., 1990; Öztop, Saraydın, & Çetinus, 2002).

Hard and soft acids and bases explain that bonds between atoms with similar rating e.g. a hard acid combined with a hard base are the strongest. In this concept, metal ions such as K^+, Ca^{2+}, Mg^{2+} and Fe^{3+} are classified as hard Lewis acids, whereas monovalent metal ions such as Ag^+ and Cu^+ are categorized as soft Lewis acids. The transition metal ions, Co^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+}, are considered "borderline acids". These metal ions are most often employed in IMAC (Immobilized metal affinity chromatography). The chelated metal ions show variations in affinity toward proteins, which can be predicted using hard and soft acids and bases. There are three major types of ligands. Those containing oxygen (e.g. carboxylate), aliphatic nitrogen (e.g. asparagines and glutamine) and phosphorous (e.g. phosphorylated amino acids) are classified as hard Lewis bases. Ligands containing sulfur (e.g. cysteine) are classified as soft bases. Those containing aromatic nitrogen (e.g. histidine and tryptophan) are considered borderline bases. The borderline acids, containing Co^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}, coordinate favourably with aromatic nitrogen atoms (borderline bases) and also with sulphur atoms (soft bases) (Pearson, 1963; Ueda et al., 2003).

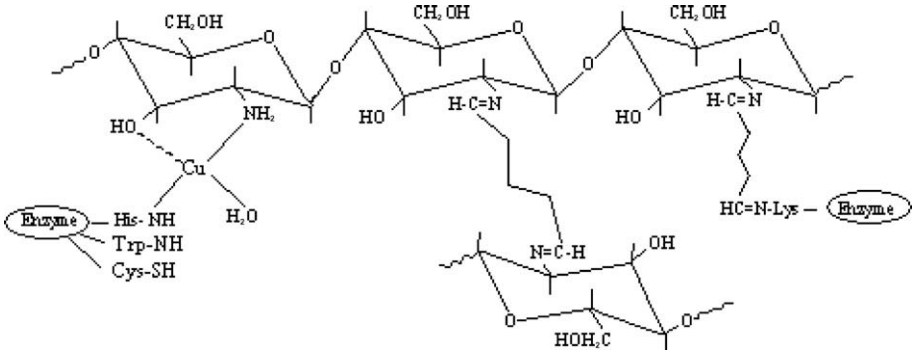
Histidine is the amino acid with strongest affinity for metal ions. Histidine, and also tryptophan and cysteine resudies show strong interactions with metal ions, which is important in binding of protein. In interactions between immobilized Cu^{2+}, Ni^{2+}, Co^{2+} or Zn^{2+} and amino acid residues on protein surfaces, imidazolyl, thiol and indolyl functional groups are the main targets for the metal ions (Arnold, 1991; Ueda et al., 2003).

The FTIR spectra of the chitosan pretreated glutaraldehyde (Fig. 2A) show adsorption bands at 910 and 1153 cm^{-1} and these bands are assigned to the monosaccharide structure in polysaccharide. There was a significant adsorption band at 1574 cm^{-1}, which can be attributed to the chacteristic peak of C—N formed from the crosslinking reaction between chitosan and glutaraldehyde. Weak adsorption band between 3200 and 2500 cm^{-1} indicates a chelate structure. In the absence of copper (Fig 2(A) a band in 2876 cm^{-1} was observed and upon addition of copper the band shifted to 2927 cm^{-1} (Fig. 2B and C) both in the presence and absence of catalase. Bovine catalase (Pdb. Id. Cat. 7) is in mainly β-sheet form and the enzyme has several histidin and tryptophan residues (Fita & Rossman, 1985). Previous studies in the literature showed that β-sheet structure was observed at 1625 cm^{-1} (Hirs & Timasheff, 1986). It was shown that this band intensity increased in the presence of copper and catalase. This increase indicates binding of the protein to the matrix. In addition to this, in Fig. 2C an adsorption band appeared at 1600 cm^{-1}, which can be attributed to the formation of C=N because of the imine reaction between amino groups from enzyme and aldehyde groups in glutaraldehyde.

In this study, proteins can interact with Cu-Ch from various sides. Firstly, ε-amino group of Lys residues on protein surfaces can bind free aldehyde groups of glutaraldehyde. Accordingly, imidazolyl, thiol and indolyl groups of His, Cys, Trp (respectively) residues on protein surfaces can coordinate with Cu^{2+} adsorbed on chitosan beads. This possible immobilization mechanism is illustrated in Scheme 1.

3.3. Kinetic studies

Kinetics of the activity of free and immobilized catalase were investigated at various concentrations (2.5–20.0 mM) of hydrogen peroxide (in 10 mM phosphate buffer at pH 7.0) as a substrate. These data were plotted according to Lineweaver–Burk and kinetic parameters, apparent *K*m and *V*max, calculated from the graphs (Table 2). The value of apparent *K*m was 18 mM whereas the *V*max was calculated as 4800 μmol (mg protein min)$^{-1}$ for immobilized catalase on chitosan beads (Ch-CAT) at 35 °C. The apparent *K*m value was 53 mM and the *V*max value was 18450 μmol (mg protein min)$^{-1}$ for immobilized catalase on Cu^{2+} adsorbed chitosan beads (Cu-Ch-CAT) at 35 °C. The value of *K*m was 35 mM and the *V*max was calculated as 32000 μmol (mg protein min)$^{-1}$ for free catalase at 35 °C. As expected, the *K*m and *V*max values were significantly affected after immobilization on chitosan beads. The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure or by lower accessibility of the substrate to the active site of the immobilized enzyme (Kennedy & Melo, 1990). This *V*max of immobilized catalase was lower than that of free catalase. When the *V*max values of Ch-CAT and Cu-Ch-CAT were compared, it was found that *V*max value of Cu-Ch-CAT was four fold higher than that of the Ch-CAT. Because, the amount of immobilized protein of Cu-Ch-CAT was higher than that of Ch-CAT, naturally, it was found that *V*max value of Cu-Ch-CAT was higher. On the other hands, in a previous study we found that *V*max and *K*m values of dye ligand chitosan catalase system were 14250 (μmol/mg protein min) and 41 mM respectively (Çetinus, Öztop, & Saraydın, 2007). These values are



Scheme 1. Possible immobilization mechanism of catalase on Cu^{2+} adsorbed and glutaraldehyde pretreated chitosan.

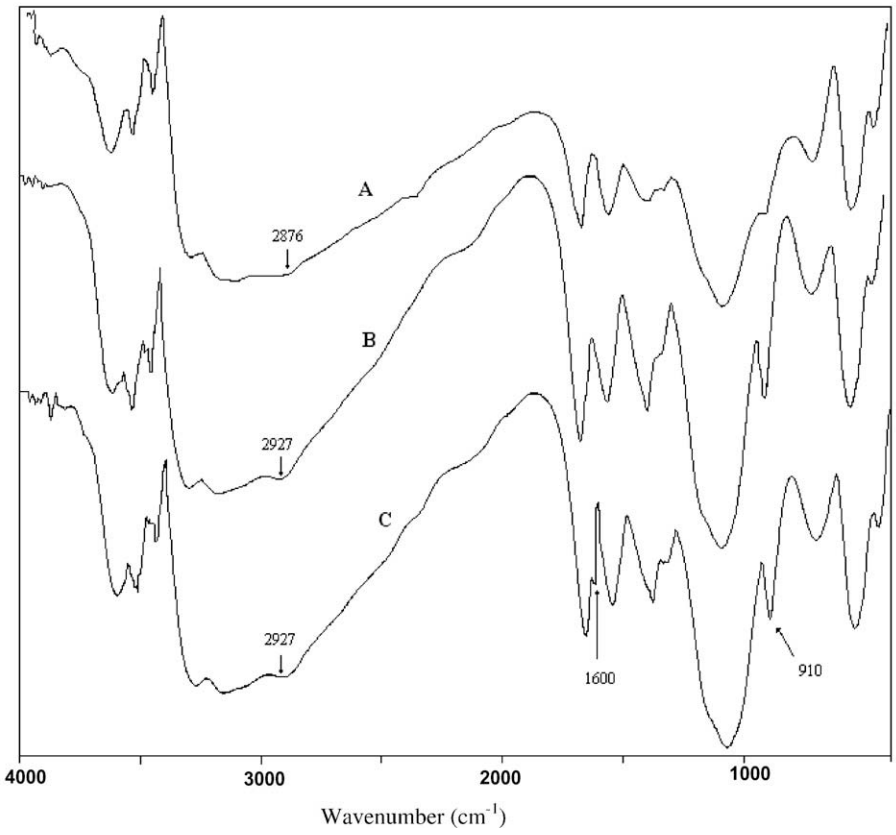


Fig. 2. FTIR spectra of Ch (A), Cu–Ch (B), Cu–Ch–CAT (C).

lower than that of our current system. So, we can say that, immobilization system with a metal ion bridge is more effective than dye bridge immobilization system. A similar result involving change in *K*m and *V*max values of enzyme after immobilization has been reported by Chatterjee, Kumar, and Sanwal (1990); Solas, Vicente, Xavier, and Legaz (1994), Krajewska et al. (1990), Çetinus and Öztop (2003); and Tükel and Alptekin (2004).

3.4. The temperature profiles and thermal stability of catalase

The temperature dependence of the activities of the immobilized catalase and free catalase was studied in 50 mM phosphate buffer (pH 7.0) at of 25–55 °C and temperature profiles of immobilized catalase are shown in Fig. 3. The optimum temperature was 35 °C for Ch-CAT and free catalase. The optimum temperature range for Cu-Ch-CAT was 25–35 °C. In this study, optimum temperature range for Cu-Ch-CAT was higher than that of Ch-CAT.

Thermal stability was investigated by incubating immobilized catalase and free catalase at temperatures ranging from 25 to 65 °C in 50 mM phosphate buffer (pH 7.0) for five hours and then determining the activity at optimum reaction temperature. Cu-Ch-CAT showed high stability at 25–35 °C. Free catalase and Ch-CAT showed high stability at 25 and 35 °C, respectively. There was no activity loss for the Cu-Ch-CAT at 25–35 °C (data not shown). The authors demonstrated that the thermal stability of enzymes may be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint (Martinek, Kilbanov, Goldmacher, & Berezin, 1977).

The conformational flexibility of the enzyme can be affected by immobilization (Kennedy & Melo, 1990; Abdel-Naby, 1993). Immobilization of catalase on copper adsorbed chitosan beads caused an increase in enzyme rigidity which is commonly reflected by increase in stability towards denaturation by raising the temperature (Jiang & Zhang, 1993). Immobilization of catalase especially by using metal ion also provides a means for protein stabilization, although Vmax values of immobilized catalase on Ch and Cu-Ch beads were lower than that of free catalase.

3.5. The operational and storage stability of catalase

When comparing performance of immobilized biocatalysts, intended for preparative or industrial use, characterization of their operational stabilities is very important. The operational stability

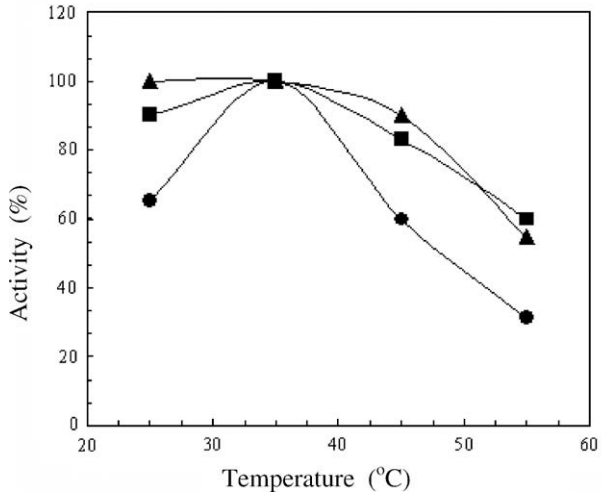


Fig. 3. Effect of temperature on the activities of catalase. (■) Free catalase, (●) Ch–CAT and (▲) Cu–Ch–CAT. Catalase activities were determined in 50 mM phosphate buffer (pH 7) of various temperature values.

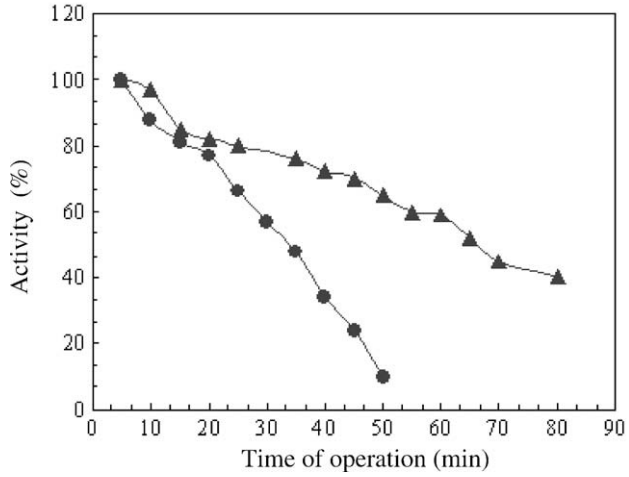


Fig. 4. Operational stability of immobilized catalase (●) Ch–CAT and (▲) Cu–Ch–CAT. Enzyme activity determined at 35 °C in 50 mM phosphate buffer (pH 7).

of immobilized catalase in the current study was evaluated in repeated batch process. Fig. 4 shows the effect of repeated use on activity of immobilized catalase. When the operation time increased, immobilized catalase activity decreased gradually. These results could be explained by the inactivation of the enzyme caused by the denaturation of the protein and the leakage of protein from the support upon use. However, the Cu-Ch-CAT showed higher operational stability than Ch-CAT. This brings an advantage over using immobilized catalase.

The storage stability of immobilized catalase was investigated. The storage stability of Ch-CAT and Cu-Ch-CAT was approximately the same. At 5 °C, free enzyme lost about 50% of its activity within 15–20 days and lost its remaining activity within 70 days, whereas Ch-CAT and Cu-Ch-CAT lost about 50% of their activity within 60 and/or 70 days. In addition, Ch-CAT and Cu-Ch-CAT protected about 50% of its activity after 70 days (data not shown). The decrease in activity was explained as a time-dependent natural loss in enzyme activity, and this was prevented to a significant degree by immobilization. Half life of immobilized catalase on both Ch and Cu-Ch beads was very high. Also this immobilization brings another advantage by increasing enzyme stability as judged by storage stability experiments.

3.6. Desorption and regeneration of Cu-Ch-CAT

Desorption of catalase from Cu^{2+} adsorbed chitosan beads was carried out in a batch system. Cu-Ch-CAT preparation was placed within the desorption medium containing 0.1 M NaSCN (pH 8.0) at room temperature for 2 h. Then, the same chitosan beads were used for Cu^{2+} desorption. Adsorbed Cu^{2+} were stripped from chitosan beads by the addition of 50 mM EDTA (ethylenediaminetetraacetic acid). After reloaded with Cu^{2+}, the chitosan beads were reused for immobilization of catalase. To investigate the reusability of the Cu^{2+} adsorbed chitosan beads support, the adsorption-regeneration cycle was repeated five times using the same chitosan beads. After repeated use the adsorption capacity was decreased around four percent (data not shown).

4. Conclusions

Cross-linked chitosan beads were prepared and copper ions were adsorbed on their surface. Adsorption of Cu^{2+} onto chitosan is found to be a Langmiur type isotherm in the Giles adsorption classification system. The present work has provided quantitative information on the binding characteristic of Cu^{+2} with chitosan.

To determine the binding isotherms, Langmiur linearization method was used. Binding parameters were evaluated. *Ki* value was 32.0 L mol^{-1}. This value shows that the interaction is very strong between Cu^{2+} and chitosan. Equilibrium constant was 1520 L mol^{-1}. This value of K indicates that Cu^{2+} has been adsorbed very efficiently by the chitosan. The monolayer capacity of chitosan is 0.020. The value of θ was 0.932. The value shows that chitosan is completely saturated by Cu^{2+}.

Adsorption free energy of the Chitosan/copper binding system was calculated (-18 kJul mol^{-1}). This value shows that the binding of Cu^{2+} onto chitosan spontaneously ($\Delta G < 0$).

Immobilized protein amount and maximum reaction velocity for Cu-Ch-CAT were higher than that for Ch-CAT. In both immobilization situation although, activities of immobilized catalase were lower than that of free catalase, Cu-Ch-CAT showed a high temperature stability, operational stability, and storage stability. Furthermore, it can be said that this copper adsorbed chitosan beads can be used as chromatographic column resin in protein purification in IMAC. The present work demonstrated the potential of cross-linked chitosan beads for metal sorption and enzyme immobilization.

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