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STABILIZATION OF ALKALINE PHOSPHATASE BY CHEMICAL MODIFICATION OF CARBOXYMETHYLCELLULOSE

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LIST OF SYMBOLS

(-)	Covalent chemical bond
ΔG_i	Free energy of inactivation
>	Greater than
<	Less than
+	Plus
(·)	Non-covalent association

LIST OF ABBREVIATIONS

ABH	Blood Group system
ADH	Antidiuretic Hormone
ALP	Alkaline Phosphatase
APAAP	Alkaline Phosphatase Monoclonal Anti-alkaline Phosphatase
B-ALP	Bone Alkaline Phosphatase
CA	Cellulose Acetate
CN	Cellulose Nitrate
EDTA	Ethylenediaminetetraacetic Acid
E coli	Escherichia Coli
GH	Growth Hormone
GOx	Glucose Oxidase
GCAP	Germ Cell Alkaline Phosphatase
HMW	High Molecular Weight
HBV	Hepatitis B Virus
NSAP	Nonspecific Alkaline phosphatase
PLAP	Placental Alkaline Phosphatase
pNPP	para-Nitro Phenyl Phosphate
PGA	Penicillin G Acylase
PPi	Pyrophosphate
SDS	Sodium Dodecyl Sulfate
TNAP	Tissue Non-Specific Alkaline Phosphatase

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ABSTRACT

STABILIZATION OF ALKALINE PHOSPHATASE BY CHEMICAL MODIFICATION OF CARBOXYMETHYLCELLULOSE

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Alkaline phosphatase is a homodimeric metallic-hydrolase where both Zn2+ an Mg2+ are important for catalysis and stability. The study of carboxymethylcellulose CMC is widely used in many industrial aspects and laboratory due to it is a natural organic polymer that is non-toxic, biodegradable, and biocompatibility. CMC was activated with l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). CMC was converted into the active form then attached to alkaline phosphatase (ALP) to form covalently conjugated CMC with ALP. The CMC–ALP was evulated under different pH values (5, 6 and 7) and temperatures (50, 60, 70 and 80 ºC). As result of the modified ALP, stabilities were increased at both pH (5 and 7) and temperature (50 and 60 ºC). However the activity of the enzyme was decreased after 70 and 80 oC. The CMC–ALP conjugation indicates the improved effect of conjugation on the substrate affinity and catalytic performance of the enzyme due to formation of intramolecular cross-links during the multipoint covalent attachment of polymer chain CMC to the enzyme molecule. In general, that modification of enzymes by the anionic polysaccharide CMC might be a useful method for improving enzyme stability under various denaturing conditions.

Keywords: Alkaline phosphatase, Modified enzyme, Carboxymethylcellulose, Enzyme stabilization, Immobilization

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ÖZET

ALKALİN FOSFATAZININ KARBOKSİMETİLSELÜLOZ KİMYASAL MODİFİKASYONU İLE STABİLİZASYONU

Sarmad Ajeel HAZZAA

Kimya Bölümü
Yüksek Lisans Tezi

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Alkalen fosfataz, "fosforik monoester hidrolazlar" olarak adlandırılan bir grup enzim içinde yer alır, içinde hem Zn2+ ve hem de Mg2+bulunur ve bunlar kataliz ve stabilite için önemlidir. Karboksimetilselüloz (CMC) aslında karboksimetil selülozun sodyum tuzudur. Suda çözünebilen bir kimyasal reaksiyonla üretilen selülozdan türetilir. CMC'nin biyouyumluluk özelliğine sahip doğal bir organik polimer olması nedeniyle laboratuvarda ve birçok endüstriyel alanda yaygın olarak kullanılmaktadır. CMC l-etil-3- (3-dimetilaminopropil) karbodiimid (EDC) ile aktive edilmektedir. CMC alkalin fosfataz (ALP) ile birleştirilir ve CMC-ALP kovalent konjuge oluşturulur. CMC-ALP'nin stabilitesi, farklı pH değerleri (5, 6 ve 7) ve farklı sıcaklıklar (50, 60, 70 ve 80oC) altında çalışılmıştır. Elde edilen sonuçlara göre, CMC-ALP'nin stabilitesi hem pH 5 ve 7'de hem de 50 ve 60oC sıcaklıkta artmıştır. Bununla birlikte enzimin aktivitesinin 70 ve 80oC den sonra azaldığı görülmüştür. CMC-ALP konjugasyonu, enzimin substrat aktivitesini ve katalitik performansını CMC'nin enzime kovalent ve çapraz bağlanmaları sayesinde arttırmıştır. Genel olarak, enzimlerin bir anyonik polisakkarit olan CMC ile modifikasyonu çeşitli denatüre edici koşullar altında enzimin stabilitesini arttırmak için yararlı bir yöntem olabilir.

Anahtar kelimeler: Alkaline fosfataz, Modifiye enzim, Karboksimetilselüloz, Enzim stabilizasyonu, İmmobilizasyon

CHAPTER 1

INTRODUCTION

1.1 Literature Review

Cross-linked sodium carboxymethylcellulose (CMC) partial toxicity information on itself the probability cross-linking would make possible decrease in any ability toxicity.

Cross-linked sodium carboxymethylcellulose was originally advanced As a pill disintegrate for medicinal products and dietary nutrients complements assafe (GRAS) status to be used in nutritional supplements [1], [2], [3].

The literature gives the key results exposed that chemically modified viscose fibers (cellulose II) have another way from cellulose wood pulp and cotton (cellulose I).

Adsorption of CMC onto viscose fibers become intentional with spectroscopy and x-ray photoelectron spectroscopy (XPS), and determined modifications at the surface of the viscose. The functionalization of CMC cause clear adjustments in the molecular shape of CMC seeming as an advance in nitrogen content material. [4]. stabilization is one of the key elements making use of for enzymes in pharmaceutical and biotechnological fields.

Chemical modification of enzymes by multipoint attachment CMC macromolecular substances employed as a method to improving the stableness and practical houses of enzymes. There are numerous previous research related the chemical amendment of various enzymes (trypsin, α-amylase, glucose oxidase, inverses and Escherichia coli penicillin) by the anionic polysaccharide carboxymethylcellulose [5], [6], [7], [8], [9].

1.2 Objective of the Thesis

In this assignment it was evaluated that the use of carboxymethylcellulose (CMC) for chemical change of alkaline phosphatase (ALP). The outcomes of different pH and temperature values on the enzyme stability were evaluated by using incubating local alp and conjugated CMC-ALP.

1.3 Hypothesis

CMC-ALP conjugate essential for several industrial biomedical and biotechnological applications.

CHAPTER 2

GENERAL INFORMATION

Alkaline phosphatase (ALP) is catalyzes the hydrolysis and transphosphorylation of protein and non-protein substrates contain of a collection of isoenzymes that are diffuse in mammalian tissues (Figure 1) [10], [11].

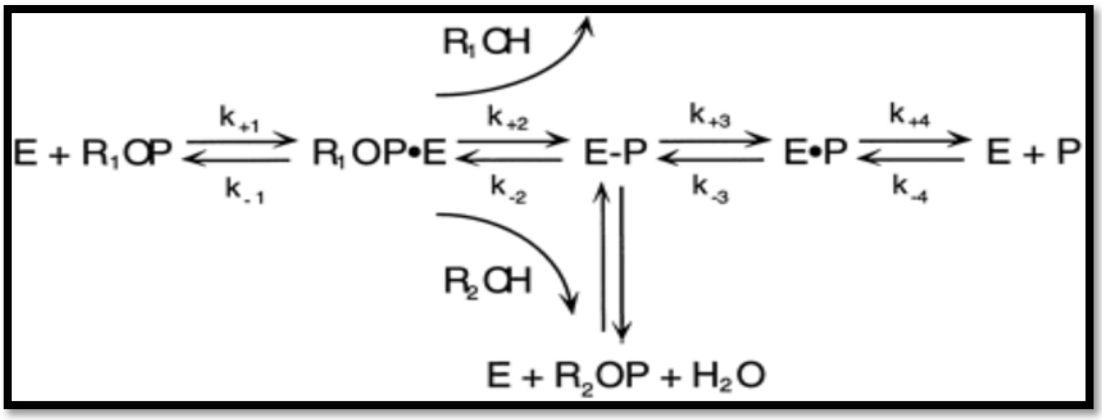


Figure 2.1 General pattern of ALP – catalyzed hydrolysis of phosphate substrates [12]

Alkaline phosphatases are demoted from diverse sources (tissues of different organs, microorganisms, connected tissues and body of invertebrates and vertebrates; human beings and animals).

ALP is pronounced in numerous tissues, such as kidney, bone, and liver and its activity is frequently considered as significant biomarker in medical diagnosis [13].

ALP interest is clearly accelerated in a few human sicknesses, together with liver diseases (hepatitis, obstructive jaundice, most cancers) and bone illnesses (osteomalacia, Paget's disorder, osteoblastic, bone most cancers) [14].

ALPs isoenzymes relying on their supply of isolation. They may be regularly divided into four companies, three of them are observed solely in placenta, intestinal, and germ cells. The fourth isoenzyme known as tissue non-particular alkaline phosphatase, due to its wide tissue circulate. ALPs from renal tissues, hepatic, skeletal, constitute this organization of indicated enzymes [15], [16].

2.1 Alkaline phosphatases (ALPs)

2.1.1 Source of Alkaline phosphatase

First identified ALPs was Suzuki [17]. "Alkaline phosphatase phosphomonoester it is far a homodimeric enzyme with a molecular weight of about one hundred 160 kDa and every one active site place contains three metal ions, one magnesium ion also two zinc ion one magnesium ion all necessary for interest enzymatic" [18], [19].

In addition to arysulfatasesare and phosphoglyceratemutates all metalloenzymes, which belong to identical superfamily. The enzyme, that may hydrolyze fructose 1-6 diphosphate and glycerophosphate, become found in maximum amount in intestinal mucosa. Phosphatases contain of an abundant and complex institution of enzymes that discover in lots of organisms both eukaryotes and prokaryotes. "The determined in extracellular fluids and cells and their role to stimulate the hydrolysis of virtually each phosphomonoester to current inorganic phosphate and the analogous alcohol, sugar or phenol, and catalyzes trans phosphorylation reactions in occurrence of huge attention of a phosphate acceptor". In nature alkaline phosphatases are exist in lots of organisms. The enzymes existing in fungi; microbes are highly adequate in mammals and fish while missing from higher plant life" (Figure 2). In mammals, tissues with excessive concentrations of alkaline phosphatase encompass intestine, kidney, placenta, bone, liver, lung, and spleen even that alkaline phosphatase is plentiful inside the tissues which are concerned with the delivery of nutrients, often being present in secretory organs and developing tissues [20], [21].

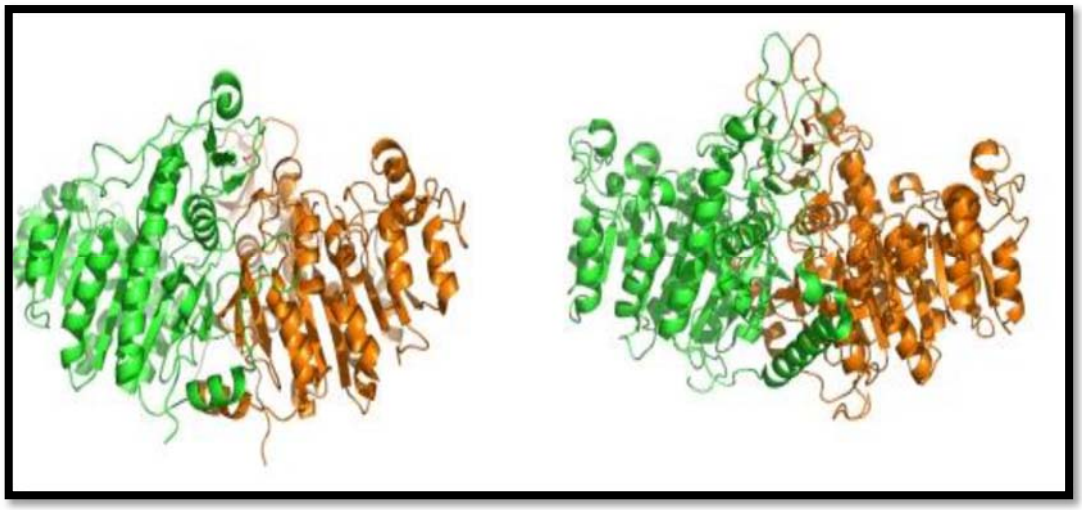


Figure 2.2 Diametric ALP of (left) Escherichia coli and (right) human placenta [22].

2.1 Physiology of Alkaline phosphatase

ALPs contain four genes in human beings: “three tissue-specific ALP is enzymes, might be 90–98% homologous, and the nonspecific ALP (NSAP) that is 50% same with the others”. The tissue-particular are “the placental (PLAP), the intestinal and the germ cellular (GCAP) that position in thymus, testis, and lung. The NSAP be located in bone, liver, kidney, blood cells, and has part of its herbal substrates Pyridoxal 5-phosphate and Phosphoethanolamine” [23], [24].

The genes of those three are living in vicinal places on chromosome 2. Despite the fact, “the tissue unspecific ALP gene is located on chromosome 1 of human genome the chromosome-2. ALP genetic factor are proved to encode the C-terminus a phosphatide inositol glycan tail that is used to bind and attach to membranes” [25].

The normal level of “ALP activity in human blood serum is 44 to 147 IU/L (international units per liter) with predictable differences coming from the laboratory practicalities, the gender, "and age with children and pregnant women having normally occurred growing raises”. In tissues with excessive condensation of alkaline phosphatase like kidney, intestine and placenta, the enzyme location at the absorptive surface propose an immediate function inside the transport of nutrients across the epithelial membrane [23], [26].

Activity of ALPs deviates in different tissues from this time their “activity in serum is commonly used in clinical tests to investigate specific diseases. Such diseases are the

adult coeliac disease, the Paget‘s disease, hyperparathyroidism, and particularly disease in liver and bones” [27].

Bone alkaline phosphatase (B-ALP) is “set up in serum and somewhere else but then it is produced rarely in bone. The increase fee of youngsters turned into studied and showed to speed up afterward growth hormone (GH) therapy. Parallel with this dashing the bone alkaline B-ALP is likewise expanded, due to this conduct overall serum B-ALP activity is taken into consideration as an essential marker for bone formation and achievement index of GH therapy” [27], [28].

Formation is inspired sure embryonic calvarias concentrations while happens in vivo and at this concentrations. It was extended whilst fluoride became utilized. Moreover, “in vitro treatment of bone cells sodium fluoride supplied to boom by way of both ALP activity and bone proliferation. The ranges of alp have been measured and as compared among agencies of children and adults that suffered or no longer from lung disease” [29].

The study results excessive molecular weight (HMW) ALP was substantially better each “youngsters (n=32) and adults (n=22) in the diseased circumstance even as the liver ALP was better simplest in children suffered from lung ailment. The concentration of ALP from lung fluids has been applied broken marker of kind II cells” [30]. The serum stages of ALP get lower than ordinary for this reason the bone forming cells explicit less NSAP.

Table 2.1 The activities of ALP in human serum [31].

ALP type	Children		Adults	
	Control (n=15)	Lung disease	Control (n=30)	Lung disease
Total ALP (UI/L)	522 + 142	619+144	190+45	176+51,3
Bone ALP (%)	84,8+5,5	75,9+6,9	30,8+11,2	29,9+11,1
Liver ALP (%)	14,1+5,5	18,1+5,5	66,8+13,9	63,4+11,5
HMW ALP (%)	0,23+0,18	5,6+2,1	2,4+2,2	6,7+3,6
p<0,001	UI/mg * 10^{-3}			

ALP has also a completely essential function in “persons addressed to skeletal mineralization. Mutations in the tissue non-particular alkaline phosphatase (TNAP) encoding gene purpose hypophosphatasia, which results in skeletal absences of various tiers”. Hypophosphatasia may additionally cause several sicknesses consisting of ricket that is a genetic disease associated with the resistance of objective organ to the action of one. 25-dihydroxyvitamin D3 [29], [32].

Hypophosphatasia is associated with “high pyridoxal 5'-phosphate concentrations in plasma and deficit of ALP activity. The pyridoxine phosphate phosphatase activity in serum is below the obligation of alp” [33].

ALP is observed in regular interest degrees in serum whilst rickets is whole but it is far found in incredible amounts from the early degrees of rickets. Hence, this insufficiency considers ALP as a detection index of rickets. Moreover, NSAP is alleged to modify the production of vitamin B-6 as the metabolism of the diet deviated from the everyday one after the inactivation of the tissue NSAP encoding gene [34], [35].

2.1.3 Structure -Function Relationship of ALP

The roles of metal ions in metalloenzymes include “direct sharing in catalysis stabilization of protein structure and regulation of enzymatic activity. Membrane alkaline phosphatase (ALP) is a steel-containing enzyme that assists as a good version for the study of metal ion interactions in enzyme catalysis. Native E.Coli ALP carries three metal ion binding web sites (two Zn^{2+} sites and one Mg^{2+} site), and studies on their roles and interrelationships have provided a few insights into the mechanism of the enzyme [36].

E.Coli alp is a zinc metalloenzyme, which can be activated by way of magnesium ion. Removal of the Zn^{+2} ends in loss of catalytic pastime whilst its replacement via other divalent cations (Mn^{2+}, Co^{3+} , Cd^{2+} , and Cu^{2+}) resulted in lower maximal interest. It is been proven that at the same time as low concentrations of Mg^{2+} inspired the refolding of E.Coli excessive attention without a doubt inhibited its reconstitution into the energetic form” [37].

This suggests that Mg^{2+} mediates stabilization and destabilization of the catalytically energetic shape of ALP at low and high concentrations respectively. In “E. coli ALP, Mg^{2+} is thought to have a regulatory effect on the expression of catalytic activity and maintenance of structure alintegrity of the enzyme. The specific binding of Mg^{2+} to apo-

ALP depends on both pH and the cooperative effects of Zn^{2+} binding. Mg^{2+} alone does not confer catalytic activity on ALP, but it does regulate the Zn^{2+} induced restoration of activity and perhaps, structural integrity of the metal-binding loci" [38], [39].

Mammalian ALPs are "glycoproteins are present as numerous isoenzymes in several tissues including bone, gut, kidney, and placenta (Figure 3) [19]. Like their E.coli counterpart, they are zinc metalloenzymes that may be activated by means of mg^{2+} ions; each ions being essential for catalysis and structural balance. The human genome, like different mammalian systems carries four ALP loci, one coding for the tissue Nonspecific ALP. Expressed (TNAP) in a variety of organs consisting of liver, kidney, bone, and so forth. In addition, three tissue-precise ALP (TSAP) genes coding for the intestinal, placental ALP (PLAP), and germ cell ALP (GCAP)".

The "TNAP suggests approximately 50% series resemblance with the TSAP isoenzymes. Mammalian ALPs show 25-35% collection identification with the E.coli enzyme in the ones zones of the protein supposing α-helix and ß-strand secondary structures and vital for catalysis" [37], [40].

The "catalytic residues, i.e. Asp 91, Ser 92, Arg 166, and ligands coordinating the divalent metal ions (Zn^{2+} and Mg^{2+}) are all conserved. These structural similarities suggest that mammalian ALPs may additionally catalyse hydrolysis of phosphate monoesters by identical mechanism as the E.Coli enzyme" [37].

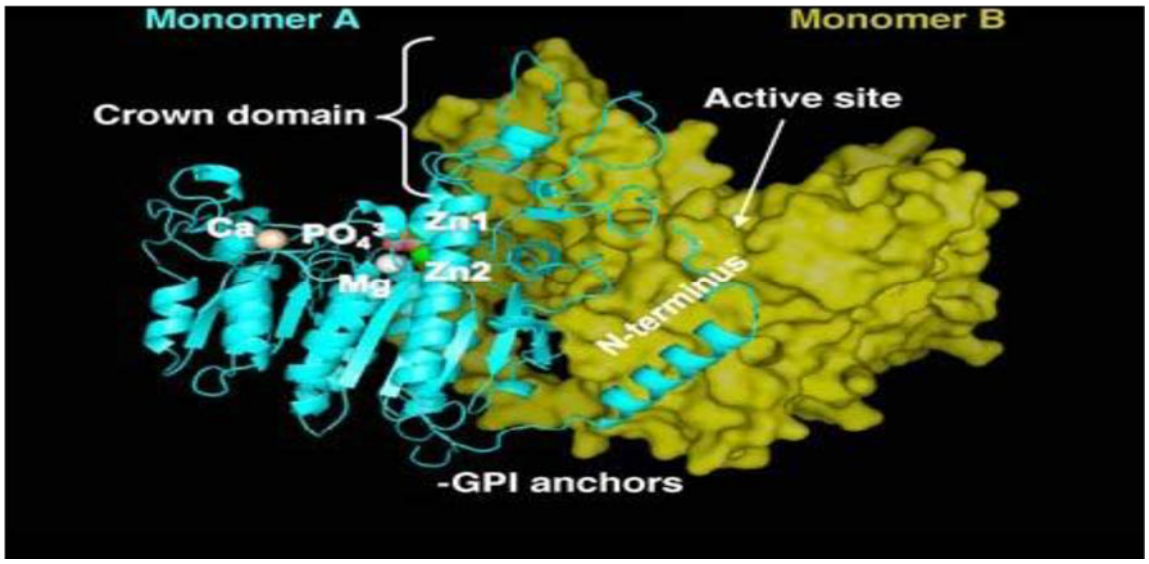


Figure 2.3 Three-dimensional structure of human placental alkaline phosphatase [19]

2.1.4 Inhibition elements of ALP

There are four modules of ester that may “act unspecifically as inhibitors of ALP. Some inhibitors are ethylenediaminetetraacetic acid (EDTA) dipyridyl and which can be metal-binding sellers, zinc and beryllium chlorides that are inhibitory steel salts, amino-binding dealers as keten, phenyl isocyanate, nitrous acid and formaldehyde and inorganic phosphate that is aggressive inhibitor”. Escherichia coli and human-placental even though Pi may be a substrate additionally for an inhibitor below particular situations. Moreover, PPi acts also as ALPs inhibitor [41].

In conclusion, “EDTA inhibits ALP even though alp is not always liable to fluoride ion divalent cation-chelating sellers indicated that amino acids inhibited the intestinal alp that turned into extracted by using butanol” [42].

Whilst the unmarried substrate ALP was inhibited by “using phenylalanine uncompetitive, the Pi acted as a competitive inhibitor and the law of extracellular ALP pastime become claimed to be the role of inorganic phosphate the inhibitory position of l-phenylalanine, at the rat intestinal ALP.

The inhibition diploma confirmed to be pH-dependent with a variety from 0 to 66 % and pH between 7 - 8 and 10 - 4. The inhibition had a height at pH 8.7 for ß-glycerophosphate and 9.2 for phenyl phosphate independently of the presence of the inhibitor” [43], [44].

2.2.5 Applications of ALP

Alkaline phosphatases can also doubtlessly be working as healing agents and therapeutic aims and show numerous uses in clinical medicinal drug and in biotechnology. An example of its use in important in a way for labeling monoclonal antibodies. The procedure known as “the alkaline phosphatase monoclonal Anti-alkaline phosphatase (APAAP) technique, gives excellent immunocytochemical labeling of tissue sections and mobile smears, comparable in clarity and depth to that carried out with immunoperoxidase labeling. For that reason, the APAAP technique is mainly suitable for labeling cellular smears (for both cytoplasmic and surface-membrane antigens) and for detecting low numbers of antigen bearing cells in a specimen (e.g. Carcinoma cells in a malignant effusion)”. The method became also applicable to the detection of antigenic molecules following their electrophoretic switch from SDS-polyacrylamide gels to nitrocellulose sheets ("immunoblotting") [45].

Another scientific use is the utility of "alkaline phosphatase to label HBV (Hepatitis B virus) DNA as probe to discover the HBV DNA in hepatitis serum". The alkaline phosphatase is "coupled with polyethyleneimine using p-benzoquine as cross-linking reagent. The changed phosphatase is then covalently connected to single strand DNA and the DNA enzyme complex was tested for blot hybridization, after hybridization and incubation with a substrate answer. Sequences complementary to the probe can be imagined directly in only 1 h as opposed to the 32 P labeled probe [46].

This experiment certified the virus DNA detection method is sensitive, specific, rapid, safe, economical, and clinically useful. A different DNA detection method was advanced in which chemiluminescent substrates are employed. Chemical or enzymatic elimination of a protecting group from stable dioxetanes produces an unsettled aryloxide dioxetane, It has been found a basically, beneficial chemiluminescent substrate for alkaline phosphatase, phenylphosphatesubstituted dioxetane. With this machine, the luminescent response may be used for ultrasensitive detection of alkaline phosphatase-related antibodies and DNA probes [47].

Alkaline phosphatases also can be beneficial for industrial waste treatment: the "p-nitro phenyl phosphate activity examination for this enzyme changed into modified to be used in freshwater sediment (Figure 4), research indicated that the restoration of purified alkaline phosphatase action turned into 100% efficient in sterile freshwater sediments whilst optimized incubation and sonication conditions had been used". Massive correlations between phosphatase and total viable cellular counts, as well as "adenosine triphosphate biomass, cautioned that alkaline phosphatase pastime has utility as a trademark of microbial population density and biomass in clean water sediments" [48].

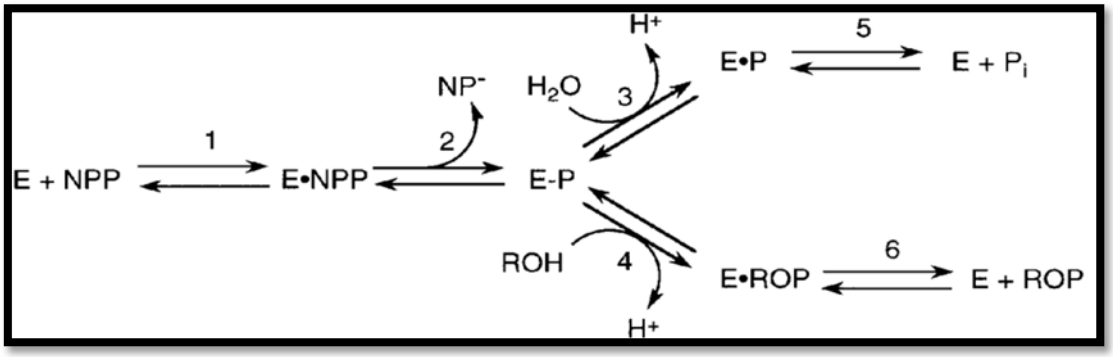


Figure 2.4 The reaction mechanism of human PLAP includes non-covalent association (.) and covalent chemical bond (-) [49].

2.1.6 Reasons of an expanded serum alkaline phosphatase

The diagnosed motives of excessive serum AIP incorporate hepatic disorders, skeletal ailment, intestinal sickness, tumours, pregnancy thyrotoxicosis the ingestion of anticonvulsant capsules and rheumatoid arthritis [50].

2.1.6.1 Skeletal Disease

Alkaline phosphatase is found in “relationship with osteoblasts which might be felt to be the source of the raised tiers of alkaline phosphatase determined in developing youngsters, in addition to in an expansion of bone disorders along with rickets, Paget's sickness, hyperparathyroidism, and osteoblastic bone secondaries” [51], [52].

Estimations of "serum alkaline phosphatase" have been found more advantageous in “the diagnosis of osteomalacia following gastrectomy by some workers, which should obviously indicate the presence of 'bone' type ALP in the serum of patients with osteomalacia even when the total serum ALP level is only slightly elevated. Various techniques to be had for this purpose, warmth inactivation research are the only to appoint but recent claims of the ability to quantitate more or less the amount of bone enzyme in serum by way of electrophoretic way are promising” [53], [54].



Figure 2.5 Patients with osteomalacia

2.1.6.2 Hepatic Disorders

The connection between excessive tiers of serum alkaline phosphatase and liver sickness has been recognized for periods as has the classical connotation that excessive serum levels of ALP are determined in "obstructive jaundice", even as in "poisonous" or "catarrhal jaundice" handiest moderate elevations are located that obstruction to

biliary out drift results in a growth within the ALP activity of the perfusate, suggesting multiplied formation of the enzyme inside the liver [55], [56].

At the same time as it is proper that more than 80% of patients with extra hepatic obstruction have “a serum alkaline phosphatase of greater than 30 KA units per 100 ml, up to 400% of patients with hepatitis additionally enzyme tiers”. Surpass there are foremost theories to provide an explanation for the elevations of serum alkaline phosphatase in liver disease. The retention theory postulates that during biliary obstruction there is a failure of secretion through the biliary tract of alkaline phosphatase' regurgitation' communications between the sinusoids and the "bile canaliculi" [57], [58].

2.1.6.3 Intestinal disease

Healthy control public of differing age companies and of recognized blood agencies and secretor reputation has been studied. “Observations of elevated levels of intestinal ALP” in ailment each fasting and after a preferred fat meal, a few cirrhotics had extended quantities of intestinal alkaline phosphatase in their serum there was a much broader variety of values amongst the cirrhotics (10-80% intestinal factor [59].

Approximately one third of cirrhotics had high tiers of intestinal ALP and in such a grossly elevated levels were visible (Figure 6). Most of this “organization with multiplied intestinal alp ranges had portal high blood pressure. Other people have assayed raised stages of intestinal ALP in fasting blood electrophoretically that allows you to diagnose cirrhosis; it show that the ranges of intestinal ALP reached in serum following a fatty meal are genetically controlled, being related to the ABH device and the abo blood corporations in sufferers with cirrhosis, as in everyday” [60].



Figure 2.6 Patients with cirrhosis

2.1.6 Tumours

Alkaline phosphatase has been mentioned in ramification of tumours and there is resistant that tumours may additionally secrete variation sorts of alkaline phosphatase into the flow. Lately, tumour isoenzyme called “the regan isoenzyme after the affected person in whom it changed into first determined has been remoted from the serum and tumour tissue of sufferers with a wide type of tumours”. This tumour isoenzyme recognized in “serum since it has properties practically conformable to placental ALP in being L-phenylalanine sensitive and very heat stable. It seems to represent another ectopic synthesis by tumours cells protein not normally manufactured from which the tumour originated”. The ectopic production of hormones such as ADH, and the synthesis by tumour cells of proteins foetal tissue, is well recognized [61], [62].

2.2 Enzyme Immobilization Technology

The first Enzyme Engineering convention became adopted in 1971. It describes enzymes bodily pent or localized in a certain location of space with retention of catalytic and which repeatedly and continuously. “Immobilization of biocatalysts (no longer simplest enzymes but additionally different bioactive molecules together with increase factors and hormones, cellular organelles, microbial cells, animal cells and plant) is attracting typical interest in biotechnology programs. In general, immobilized biocatalysts are greater strong and less difficult to deal with compared with their unfastened counterparts”. [63], [64].

Programs of immobilized biocatalysts comprise the production of useful compounds by stereospecific or bioconversion, the production of electricity with the aid of biological procedures, the selective therapy of specific contaminants to clear up environmental problems, non-stop analyses of compounds with excessive sensitivity and specificity, and clinical uses along with new styles of pills for enzyme remedy or synthetic organs [65], [66].

2.2.1 Immobilizing Enzymes Methods in Polymeric Carriers

Various methods have been advanced for the immobilization of biocatalysts, which might be getting used appreciably these days. Extensive variety of help materials has also been hired for enzyme immobilization methods of immobilization of biocatalysts (Figure 7) [67].

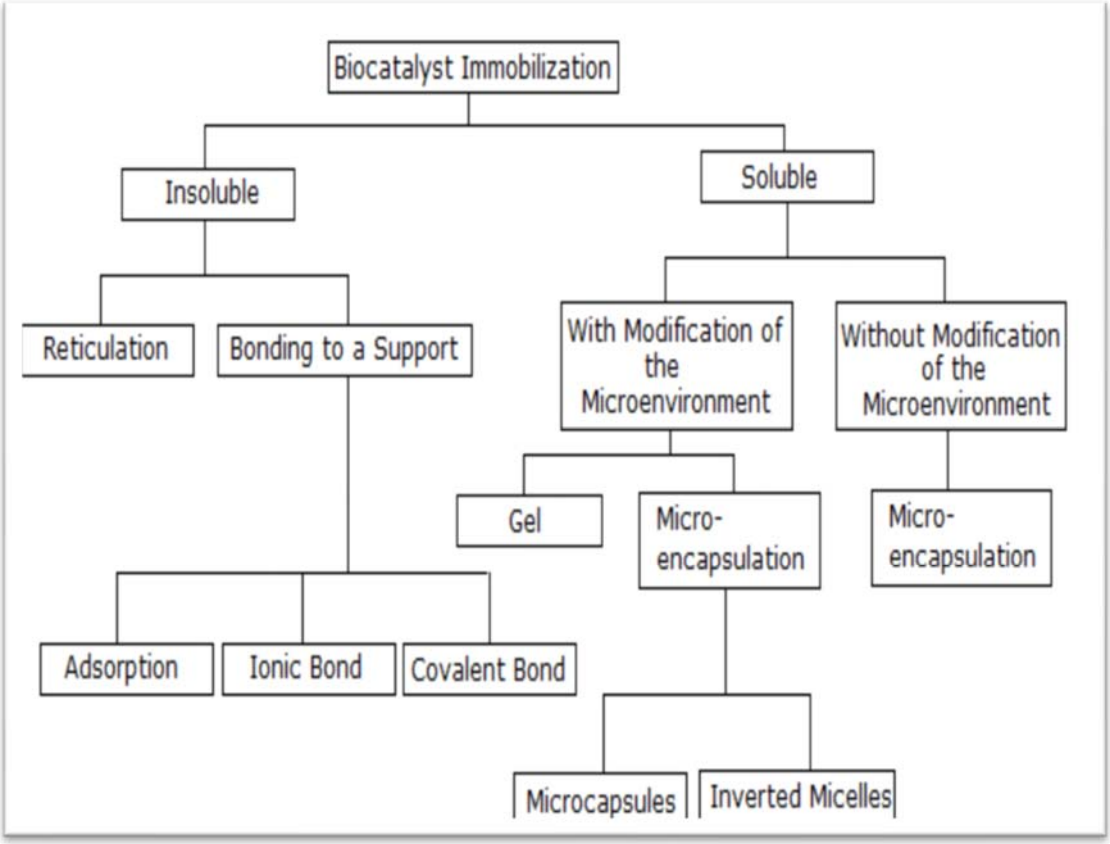


Figure 2.7 Methods of immobilization of biocatalysts

2.2.1.1 Adsorption

Enzyme immobilization by way of adsorption is the only method of “obtaining an immobilized enzyme. Principally, the enzyme is attached to the support material by using noncovalent linkages and does now not require any pre activation step of the support. The interactions shaped between the enzyme and the assist cloth might be depending on the existent surface chemistry of the help and on the kind of amino acids uncovered on the surface of the enzyme molecule”. The enzyme desorption can with ease arise by adjustments within the environment medium including pH, temperature, solvent, and ionic energy or within the case of prolonged reactions. It has “weak interactions between the guide and the enzyme which includes ionic or hydrophobic interactions, hydrogen bonding, and van der Waals forces” [68], [69].

Most of the aid materials available have sufficient floor-charge homes suitable for immobilization with the aid of adsorption. They encompass inorganic vendors “ceramic, alumina, activated carbon, kaolinite, bentonite, and porous glass”, natural artificial vendors “nylon, polystyrene” and natural carriers "chitosan, dextran, gelatin,

cellulose, starch". The technique requires strict manipulate of "the pH and ionic power, because those can alter the costs of the enzyme and the guide and consequently affect the level of adsorption". The primary advantages of adsorption are the method simplicity, the little impact at the conformation pastime of the biocatalyst, and the opportunity of regenerating inactive enzyme biocatalysts certain to a service by means of adsorption (Figure 8) [70],[71].

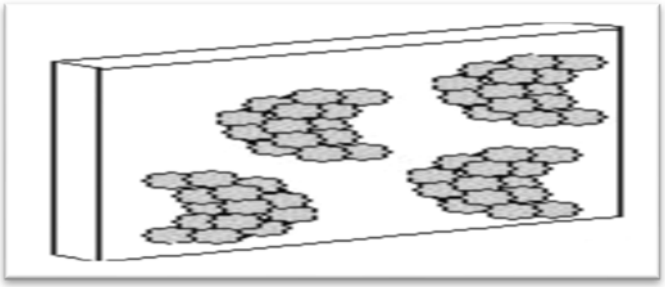


Figure 2.8 Biocatalysts bound to a carrier by adsorption.

2.2.1.2 Binding of Ionic

Immobilization by ionic binding is "based mainly on ionic binding of enzyme molecular active molecule to stable supports containing ionic costs .In this method, the amount of enzyme bound to the carrier and the hobby after immobilization depends on the nature of the service" (Figure 9) the enzyme is certain to the service. In a few instances, physical adsorption may additionally take vicinity. The principle distinction among ionic binding and physical adsorption is the strength of the interaction that is a lot stronger for ionic binding, despite the fact that less sturdy than covalent binding. The "preparation of immobilized enzymes the use of ionic binding is based on the same method as described for physical adsorption" [72], [73].

The ionic nature of the binding forces between the enzyme and the guide also relies upon on pH versions, guide price, enzyme concentrations, and temperature. The helps used for ionic binding may be based totally on polysaccharide derivatives (e.g., carboxymethylcellulose, diethylaminoethylcellulose, chitosan, dextran,) synthetic polymers (e.g. polyethylenevinylalcohol, Polystyrene derivatives,) and inorganic materials (e.g. alumina, ambertite, silicates, sepiolite, bentonite, silica gel, and many others.) [65], [67].

The immobilization with the aid of “ionic binding has the improvement that modifications within the enzyme conformation handiest occur in a small volume, ensuing in immobilized enzymes with excessive enzymatic activities. The main disadvantage is the possible interference of other ions and special attention should be paying in maintaining the correct ionic strength and pH circumstances in order to prevent their easy separation” [63].

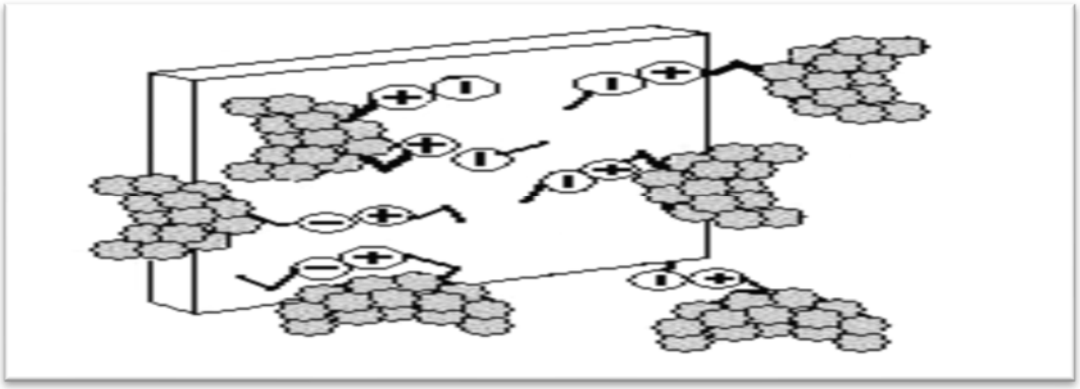


Figure 2.9 Biocatalysts bound to a carrier by ionic binding

2.2.1.3 Covalent Binding by Chemical Coupling

The covalent binding technique is primarily based at the binding of enzymes or other active molecules to a guide or matrix by using covalent bonds (Figure 10). The bond is “normally formed among a useful organization present at the help surface and amino acid residues on the floor of the enzyme. Natural polymers, which might be very hydrophilic”.

The ones which are most often involved in covalent binding are the “amino (NH_2) group of lysine or arginine, carboxyl (CO_2H) institution of aspartic acid or glutamic acid, hydroxyl (OH) institution of serine or threonine and sulfhydryl (SH) group of cysteine” [74], [65].

There are numerous response tactics for “joining an enzyme to a material with a covalent bond diazotation, amino bond, Schiff's base formation, amidation reactions, thiol-disulfide, peptide bond, and alkylation reactions)”. It is important to choose a technique with a purpose to not contain the response with the amino acids gift within the active site; two steps are worried inside the covalent binding of enzymes to assist fabric.

First, useful corporations at the support material are activated by means of precise reagents (e.g.Carbodiimide, cyanogen, bromideaminoalkylethoxysilane, epichlorohydrin, isothiocyanate and epichlorohydrin). A large variety of support materials is to for covalent binding and this substantial variety reflects the element that no perfect matrix exists [75].

The “Immobilization of enzymes via covalent attachment has additionally been set up to set off higher resistance to temperature, denaturants, and natural solvents in numerous cases. Different factors might also influence the choice of the help and some of the more critical are its price and availability”. The binding capacity “(amount of enzyme bound per given weight of matrix), hydrophilicity structural rigidity, and durability during applications. A frequently encountered disadvantage of immobilization by means of covalent binding is that it places great stress on the enzyme activity” [74].

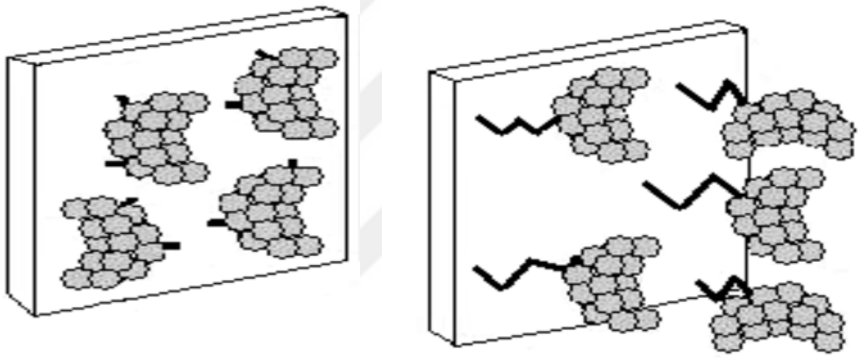


Figure 2.10 Covalent bond between biocatalysts and a carrier with and without spacer

2.2.1 Crosslinking

The crosslinking technique is based on the development of covalent bonds among the enzyme or energetic molecules by using bi- or multifunctional reagents. The character biocatalytic devices (enzymes, organelles, complete cells) are connected to one another with the assist of bi-or multifunctional reagents (e.g, glutaraldehyde, glutardialdehyde, glyoxal, hexamethylene diisocyanate, diisocyanates, toluene diisocyanate, etc.) [76].

Enzyme crosslinking involves typically the amino groups of the lysine but in occasional instances the sulfhydryl organizations of cysteine, phenolic OH groups of tyrosine, or “the imidazol group of histidine can be used for binding. Figure 11 appearances how the biocatalysts can be linked by simple crosslinking process. In addition by “co-crosslinking, in which indeclinable molecules are incorporated in the high polymer

network in order to improve. The advantages and disadvantages of a given matrix must be occupied into account when considering the suitable Procedure for a given enzyme immobilization" [65].

The primary negative aspects are the fragility of the debris produced in some cases and diffusion obstacles. For the reason that crosslinking and co-crosslinking normally contain covalent bonds, immobilized biocatalysts in this way regularly publish to modifications within the conformation with a resultant lack of pastime. The isomerization of glucose method is a "very vital model of the economic application the use of biocatalyst cross-linked with glutaraldehyde. Some of the immobilized preparations used in these large-scale processes are produced simply by glutaraldehyde treatment of bacterial cell masses that have formed fine particles" [77].

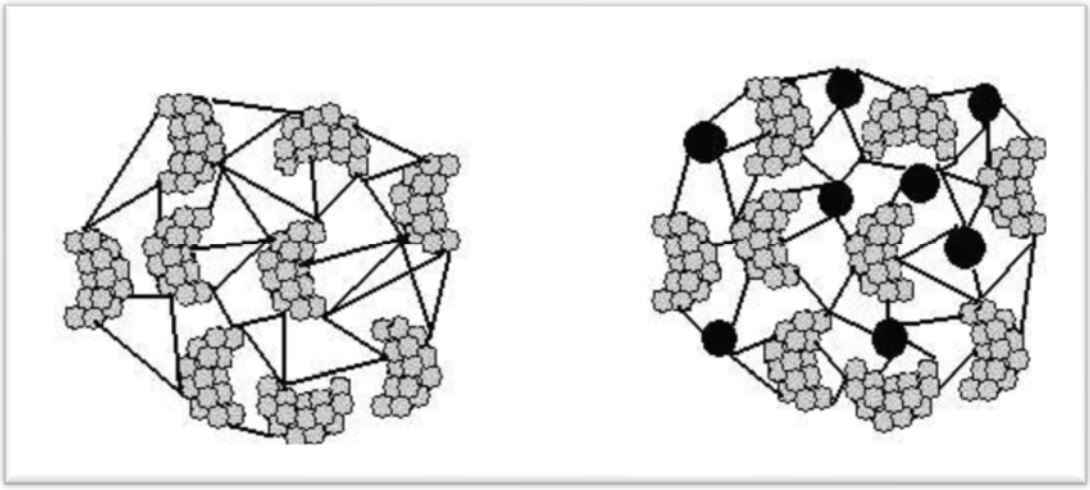


Figure 2.11 Biocatalysts immobilized by means of crosslinking and co-crosslinking with inert molecules incorporated.

2.2.1.5 Entrapment and Encapsulation

The entrapment method for immobilization consists of the physical trapping of the active components into a film, fiber, gel, coating, or microencapsulation. This method can be accomplished by mixing an enzyme or active molecule with a polymer and then crosslinking the polymer to form a lattice structure that traps the enzyme (Figure 12). Microencapsulated enzymes are formed by enclosing enzymes solution within spherical semipermeable polymer membranes with controlled porosity [78].

The benefits of this immobilization approach are the very large floor area between the substrate and the enzyme, internal a fantastically small extent, and the actual

opportunity of simultaneous immobilization. The most important risks of this technique consist of the occasional inactivation of enzyme for “the duration of microencapsulation and the high enzyme concentration required. In addition, to retain the enzyme, the pore size needs to be very low and these systems tend to be very diffusion limited” [65], [79].

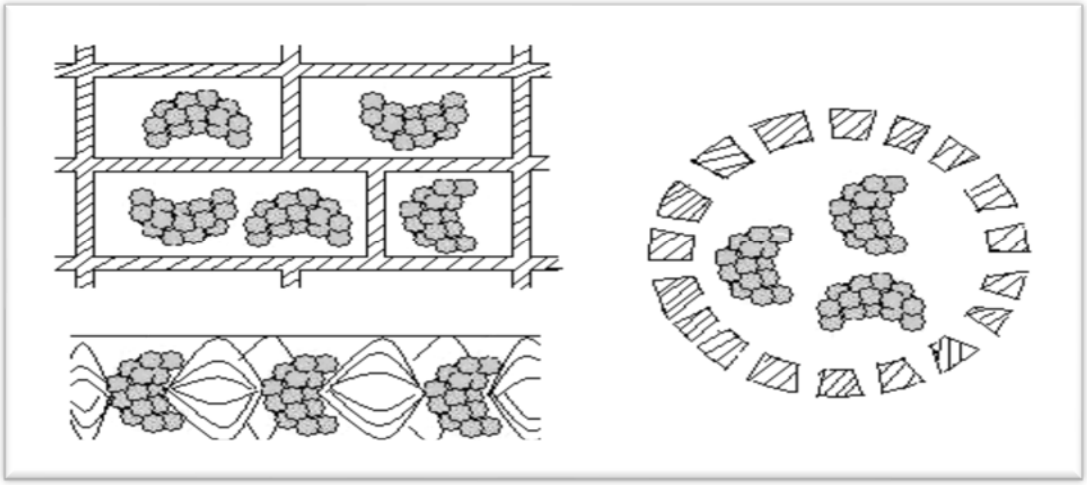


Figure 2.12 Enzyme encapsulation in a matrix, fiber, or capsule

2.3 Alkaline Phosphatase Immobilization

Alkaline phosphatase has a dimeric structure that dissociates without difficulty leading to inactivation. Immobilization by way of covalent bonds may also play crucial function in fending off dissociation of the enzyme through retaining the sub-units collectively. Covalent immobilization of enzymes normally offers the strongest linkages between enzyme and service. Consequently, the leakage of enzyme from the matrix is minimized with covalently bound enzymes [80].

Covalent binding of an enzyme to a service is common primarily based on a chemical response among the active amino acid residues placed on the enzyme surface and energetic functionalities, which might be attached to the provider floor. The covalently immobilized enzyme can be regarded as a composite, which include the components provider, spacer, linkage and enzyme [81].

To the capability of the service and/or the enzyme have to be activated earlier than immobilization and often providers are activated earlier than their use for binding enzymes (Figure 13) the physical and chemical nature of the provider strongly dictates the performance for instance activity selectivity stability and particularly the application of the immobilized enzymes obtained [80].

All through the immobilization procedure, the enzyme molecules are transported to close touch with the surface of the service and thus. The following interactions may additionally disturb the local forces that preserve the enzyme native shape, leading to modifications on the structure and characteristic of the enzyme however; it has been observed that the enzyme typically adopts a conformation stabilized by using the interplay of the enzyme and the carrier. the brand new stimulate conformation bear a similarity to the local structure of the enzyme, the immobilized enzyme is stabilized, in any other case it will be deactivated. Therefore, it is very critical to find the proper provider for a given enzyme. Epoxy- and amino-activated helps are able to form very stable covalent linkages with one of kind amino acid residues [82].

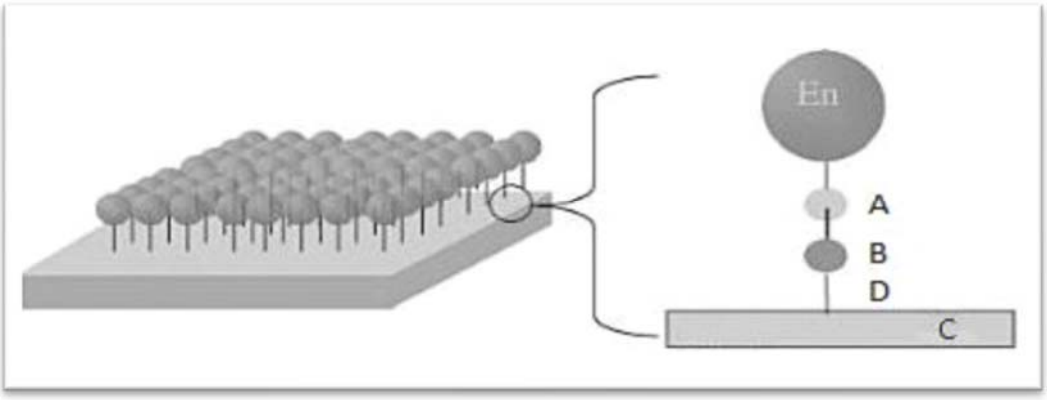


Figure 2.13 Covalent immobilization of enzyme on the carrier: (A) active amino acid residue; (B) binding functionality of the carrier; (C) carrier; (D) spacer [80].

2.4 Cellulose

2.4.1 Sources of Cellulose

Cellulose, the most plentiful renewable and biodegradable polymer, is the hopeful feedstock for the production of chemicals for their applications in various industries. Every year production of cellulose in nature is estimated to be “1011 - 1012 t in two forms, partially in a perspicuous form, for example seed hairs of the cotton plant, but mostly as hemicelluloses in cell wall of woody plants”. The adaptability of cellulose has been reconsidered as a useful structural and functional material. The environmental interest of cellulose have become even more apparent [83], [84].

Cellulose is revered as a production cloth, particularly within the shape of intact wood but also in the form of natural fabric fibers like cotton or flax. Cellulose may be derived from a diffusion of sources, which includes annual plant life, woods, microbes, and animals.

Those include “seed fiber (cotton), wooden fibers (softwoods and hardwoods), bast fibers (hemp, flax, jute, ramie), algae (valonica ventricosa), and microorganism (acetobacter xylinum). axylinum can synthesize extracellular pellicles of cellulose from glucose. a few simple marine animals which includes tunicates deposit cellulose in their mobile walls. Also cellulose, those materials additionally include hemicelluloses, lignin, and a comparable small quantity of lignin” [85].

2.4.2 Cellulose Structure

Cellulose (C6H10O5) n was first isolated and identified in 1838 since then vast research efforts have been directed towards the characterization and technological manipulation of cellulose.

Its structure contains “D-glucopyranose units, generally referred to as anhydroglucose units (AGUS) linked with ß-(1-4) glycosidic bonds, acetal bonds. In woody tissue, native cellulose chains contain roughly 10,000 of these repeat units. The ß-(1-4) glycosidic (acetal) linkage is susceptible to acid catalyzed hydrolysis but is stable in neutral to alkaline solutions” [86].

Each AGU contains “three free hydroxyl (OH) groups, the primary hydroxyl at carbon atom (C6) and the two secondary hydroxyls at (C2) and (C3). These hydroxyl groups make cellulose hydrophilic and chemically reactive. Secondary interactions at these sites promote cellulose alignment and coherence into highly oriented fibrils having crystalline and noncrystalline regions (Figure 14) repeating unit of the cellulose chain” [87].

Every cellulose chain has distinct ends analogous to unmarried pyranose unit. “Decreasing end” refers back to the hemiacetal structure resulting from ring closure at the (C1) aldehyde, and the non-lowering give up reveals the secondary hydroxyl unit on the (C4) function. However, “alkaline conditions will promote another degradation pathway where the terminal reducing sugar is cleaved, exposing the consecutive reducing sugar fate. This degradative progression (DP) is referred to the peeling reaction and while it can cause a serious DP reduction, it is not a damaging as acid hydrolysis” [88].

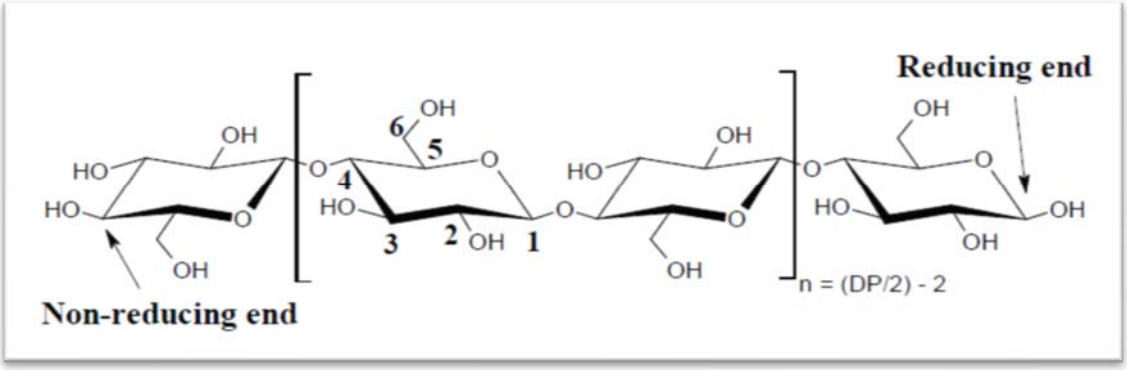


Figure 2.14 Molecular structure of cellobiose [89]

2.4.3 Cellulose Derivatives

Cellulose derivatives have been industrially great for over a century, and represented the primary industrial thermoplastic substances. Nowadays, the commercial use of these cellulosic materials is extremely broad. They represent a major material resource for "thermoplastic fibers, films, textiles, coatings, food additives, and pharmaceutical technologies. Commercial use of cellulose-based materials is expected to increase in both breadth and depth as industries look for more sustainable and environmentally friendly raw materials". Cellulose derivatives are generally categorized as two separate classes, esters and ethers [90].

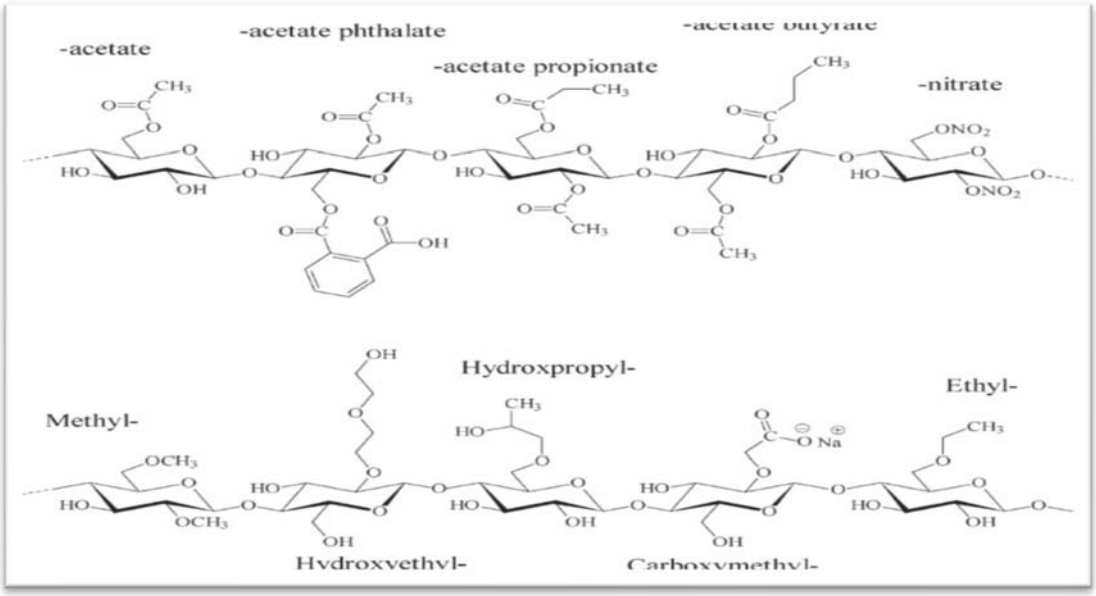


Figure 2.15 Commercially produced cellulose esters (top) and ethers (bottom) [95]

2.4.3.1 Cellulose Esters

Esters of cellulose are long known derivatives of the polysaccharide. Hydroxyl organizations present in cellulose play a vital position in its chemical conversion. The esterification response of number one and secondary hydroxyl organizations of cellulose does not fluctuate from that of other alcohols. Three OH groups gift at each glucose unit make the formation of mono- di- or triesters viable. Mutual linkages of the OH companies by hydrogen bonds inside the supramolecular structure of cellulose are partially or absolutely cleaved during esterification. The added ester organizations push the cellulose chains apart, as a result inflicting strong changes within the structure or even destroying it.

Incision of the molecule chain competes with the catalyzed esterification, but can be controlled beneath appropriate situations. Formation of cellulose esters is theoretically possible with all inorganic and organic acids. The maximum important of them are cellulose nitrate, cellulose xanthate and cellulose acetate due to their technological and commercial usage (Figure) [91], [92].

Cellulose nitrite, sulphate, phosphate, tricarbanilate, and some esters with higher carbonic acids have also won importance. Cellulose nitrate (CN) changed into the first cellulose by-product and “only industrially produced inorganic cellulose ester”. CN were commercially crucial materials for “propellant and explosive technologies, lacquers, photographic films, and molded plastics. In truth, celluloid, a CN product softened with camphor, is appeared because the first thermoplastic molding compound, and is still used these days in many industries” [93].

Cellulose Acetate (CA) is by way of a ways the maximum commercially critical cellulose derivative, and is produced as an uncooked cloth for many industrial packages. CA was “first synthesized in 1865 by means of heating cellulose in acetic acid below strain; nowadays it is manufactured with the greater reactive acetic acid anhydride and sulfuric acid catalyst” [88], [94].

2.4.3.2 Cellulose Ethers

Cellulose etherification should “occur using swollen, activated cellulose. However, for etherification, activation is conducted in aqueous alkali (NaOH) which creates swollen alkali cellulose. Cellulose ethers are soluble in a wide range of solvents, often including water and the chain length will have a large impact on the solution viscosity”.

As earlier revealed cellulose chain, peeling occurs in “alkaline media, so during the pre-etherification swelling process is carefully controlled by how long the polymer is allowed to remain in the swelling agent” [97], [98].

The etherifying agent, generally an alkyl halide or epoxide, is then added to the caustic slurry of alkali cellulose; the “ensuing substitution reaction takes place stoichiometrically as NaOH is consumed according to Williamson synthesis [95].

Ethers can also be prepared the use of simple epoxides, which with ease react with activated cellulose, in which NAOH without a doubt acts as a catalyst [95]. Further to regularly being water soluble, cellulose ethers are hydrolytically strong and are non-toxic. Hence, they have grown to be particularly vital polymers in many numerous fields. Types of substituent are crucial in figuring out cloth houses [99].

Carboxymethylcellulose

Carboxymethylcellulose (CMC), prepared with monochloroaceticacid, is the most commercially important cellulose ether with annual production over 230 kilo-tons. Commercial CMC’s in the sodium salt form, are available in DP ranges between 0.2 – 1.5. In contrast, the free acid form of CMC is water-insoluble and has very limited applications. Initially, CMC products contain residual sodium chloride salt, and sodium glycolate. Typical carboxymethylation method involving given below two competitive reactions was followed [97], [98].

$$CELL\text{-}OH + CICH_2COOH + NaOH \longrightarrow CELL\text{-}OCH_2COONa + Na\,CI + H_2O$$

Cellulose Monochloroaceticacid Sodium carboxymethylcellulose

$$CLCH_2COOH + NaOH \longrightarrow HOCH_2COONA + NaCl$$

Sodium glycolate

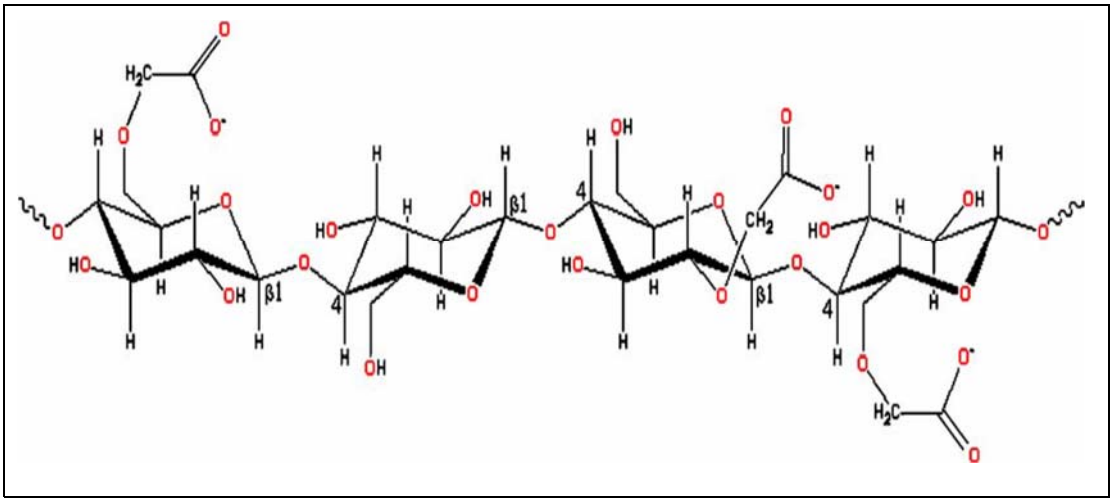


Figure 2.16 the monomeric structure of CMC [102].

In assessment, the free acid shape of CMC is “water-insoluble and has very confined applications. CMC products contain residual sodium chloride salt, and sodium glycolate. These purified CMC’s are used in detergents where they both bind with soil particles, and prevent re- precipitation onto fabrics”. Similarly, purified CMC’s (Figure16) are used in many industrial oil, water, and natural gas digging applications where they relate to rock dust. CMC purification is conducted with alcohol-water mixtures that extract the residual by products. Purified products are used in surface coatings and emulsion paint formulations as well as for pulp sizing in paper manufacturing. The highest purity CMC’s are used in pharmaceutical and cosmetic formulations, and as diet additives and stabilizers in many beverages [100], [101].

2.5 Studies on Some Enzyme Activities Modified by Carboxymethylcellulose

2.5.1 “Preparation and Functional Properties of Trypsin Modified Carboxymethylcellulose”

Trypsin from bovine pancreas changed into changed by the polyaldehyde spinoff of carboxymethylcellulose (CMC). The anionic polysaccharide CMC has “water solubility, biocompatibility and nontoxicity of many polysaccharides”, in addition to the reality that in evidently going on glycoenzymes. The carbohydrate residues feature as stabilizers of the Three-dimensional structure of the protein moiety. CMC previously activated through period ate remedy changed into connected to trypsin.

The modified enzyme contained 57% carbohydrate with the aid of weight, as a result of the amendment of 52 % of the amino organizations of the protein [103].

CMC is a linear and in part o-carboxymethylated ß-(1-4) glycan. Because of this, simplest the glucosidic gadgets wherein both hydroxyl organizations at C2 and C3 positions are not substituted this product displayed that 8 out of 15 amino businesses from trypsin have been changed with polyaldehyde–CMC. The carbohydrate content material of CMC-modified trypsin changed into calculated as 57% with the aid of weight of changed enzyme. This fee represented 237 mol of carbohydrates (as glucose) consistent with mole of trypsin inside the CMC- enzyme complicated (Figure 17).

Amino group as well as the amino corporations from lysine's might be convert into acid unsettled Schiff bases, which “yield secondary alkyl amines via NABH remedy trypsin incorporates 14 lysine residues and its N-terminal residue is an isoleucine. None of these amino acid residues participates within the active web site of the enzyme, a conventional asp102–his57–ser195 triad from mammalian serine proteases”. The rate for optimal pH and Km for esterolytic action was altered after this alteration [104].

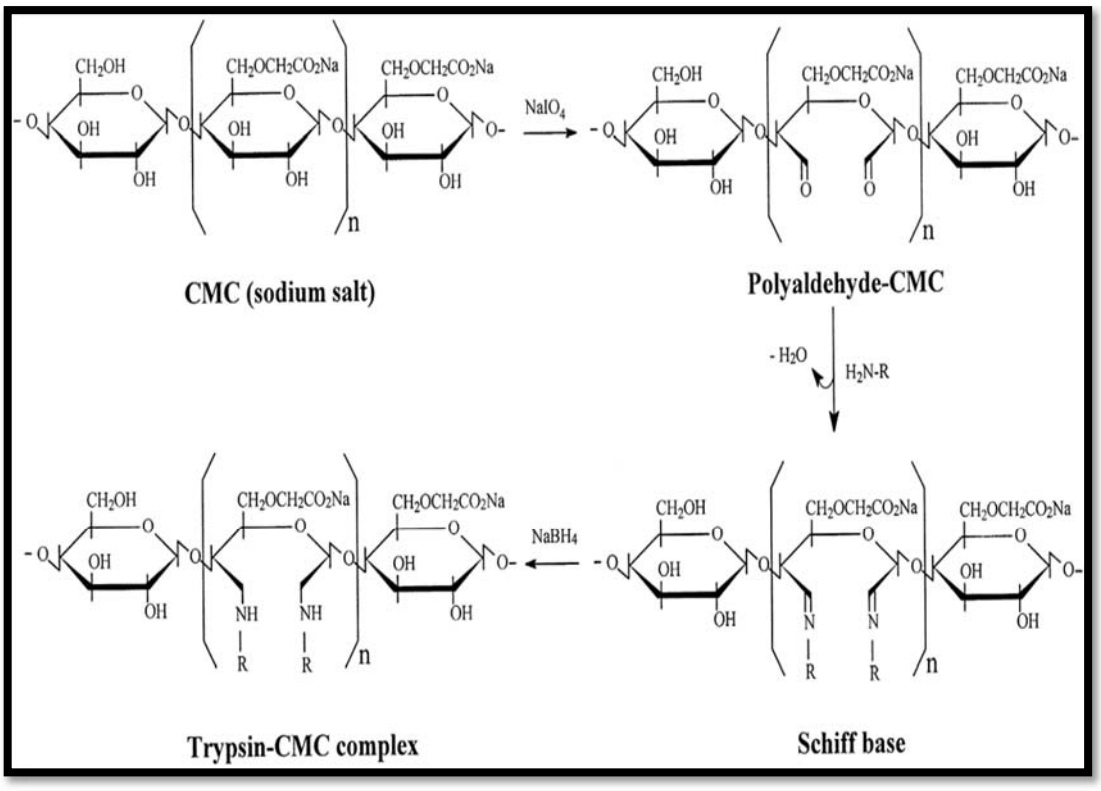


Figure 2.17 Synthesis of trypsin–CMC conjugate. H2N-R indicates protein [105]

Synthesis of trypsin–CMC conjugate. H_2N-R indicates protein [105] the “mechanism of catalysis for trypsin reveals that the His57 imidazole ring acquires a proton from catalytic Ser195 in the transition state. Consequently, one role for the conserved Asp120

is to provide electrostatic compensation for the developing positive charge in His57". In fact, site aim mutagenesis testing have demonstrated that a negative charge, adjacent to His57, is fundamental for active catalysis in trypsin [106].

Affording to this instrument, "it can be predictable that anionic groups from CMC could contribute to the electrostatic stabilization of His57 in the transition state. Then, the kinetic parameters in addition to the optimal conditions for enzyme catalysis were affected".

Conversely, "the three-dimensional structure of trypsin reveals that Lys188, which is located at the surface loop 1 near the substrate-binding pocket" [107].

The thermo stability was improved for trypsin by the conjugation with CMC. The value of temperature "50% for trypsin–CMC complex, defined which 50% of the initial activity was retained below the conditions sub. This stabilization results from the contribution of several factors within the upkeep of the active conformation of the enzyme. The most vital thing is the conformational stabilization of trypsin molecules because of intermolecular crosslinking resulting from the multipoint attachment of one molecule of polymer to numerous amino agencies from the same polypeptide chain" [108].

High degree of intermolecular cross-linking was "predestined for trypsin–CMC complex Protein aggregation plays vital role inside the thermal denaturation mechanism of trypsin. For this reason, other elements contributing to the improved thermo balance of the changed enzyme could be the electrostatic repulsion among the enzyme molecules attached to the anionic polysaccharide" [109].

Some other thing donating to the stabilization of modified trypsin is the ionic interplay among "positively charged groups on the surface of the protein and carboxylate groups from CMC". The formation of "hydrogen bonds between the covalently attached polysaccharide and the hydrophilic amino acid residues on the surface of the enzyme also are critical elements contributing to trypsin stabilization".

The relative pastime for modified trypsin was decreased from "94 % to 83 %", whilst "all carboxylate agencies from CMC have been converted into the acidic form through changing the pH from 3.0 to 2.0. In assessment, no enormous variant on the interest was located for local enzyme at this limb of pH" [110].

2.5.2 Stabilization of α-amylase by chemical Modification with carboxymethylcellulose

Stabilization is one of the key elements for applying enzymes in biotechnological and pharmaceutical felds. Protein modification through macromolecular materials has been employed. The thermo stability and pH balance had been advanced for α-amylase by this modification and realistic houses of enzymes. Carboxymethylcellulose activated with the aid of periodate oxidation changed into covalently connected to porcine pancreatic a-amylase (PPA). The unique interest of the conjugate become 54% of the native enzyme. The carbohydrate content material become intended to be 26% via weight due to the amendment of 67% of the amino companies from the protein comparison with the native enzyme. consistent with the amino acids collection of porcine pancreatic a-amylase, this end result provided that, 14 amino agencies out of 21 from native enzyme have been adjusted with dialdehyde-CMC [111].

The carbohydrate contents for the CMC- PPA complex have been taken into consideration as 62% with the aid of weight of modified enzyme.

This result represented 497 mol of carbohydrates (as glucose) per mol of protein in the CMC-enzyme conjugate. The specific activity of PPA toward soluble starch was reduced after the modification. The CMC-PPA complex retained 54% of the original activity of the enzyme. This reduction could be explained by lower accessibility of the macromolecular substrate to the active site of the enzyme, originated by the binding of a polymeric molecule to the surface of the protein. The effect of the chemical modification of PPA on enzyme thermo stability was investigated.

The thermal stability profile of CMC-modified enzyme after 10 min of incubation at one of kind values of temperature retained over 56% of the number one interest after 60 min of incubation at 55°C. In assessment, the enzymatic activity of native PPA swiftly reduced and changed into completely misplaced after 20 min of incubation at this temperature. This stepped forward thermal stability of the conjugate could be described through the maintenance of the active enzyme conformation, due to a multipoint attachment of the 2,3-dialdehyde derivatives of CMC on the protein surface Intermolecular crosslinking has in fact already been demonstrated to enhance the structural stability of a protein [112], [113].

The polyanionic nature of CMC could also support the thermo stability of CMC-modified PPA by prevention of protein aggregation through electrostatic repulsions among the conjugate molecules. Comparable stabilization was observed for CMC-modified PPA in the presence of sodium dodecyl sulfate (SDS).

Native enzyme was deactivated after 50 min of incubation in 0.3% SDS (w/v), but the CMC-PPA complex determined a residual activity of about 30% after 120 min in these conditions. PPA is a (b/a) 8-barrel enzyme (domains A and B, residues 1-403), which also contains a C-terminal domain (residues 404-496) with crystalline topology.

Since the modified estimated is a polymeric substance that has multivalent reactive sites, it may cover the surface of the protein and hold the higher order structure of PPA molecule through multiple secondary amine linkages. This freezing of the protein structure could be the main factor for stabilizing the modified enzyme, but as was illustrate above, electrostatic and hydrogen interactions between the polysaccharide and the enzyme could contribute to this stabilization [114].

2.5.3 Stabilization of glucose oxidase with cyclodextrin-branched carboxymethylcellulose

The polysaccharide used as enhancing agent for glucose oxidase (GOx) turned into prepared with the aid of “oxidizing CMC with sodium metaperiodate, pursue via conjugation to mono-6- hexylenediamino-6-deoxy-ß cyclodextrin in the presence of NaBH4. The conjugation of CMC (molecular weight = $3.0x10^4$, degree of carboxymethylation = 70%) with CD moieties produced a water-soluble macromolecular structure with a 28:1 CD-polymer ratio” [115].

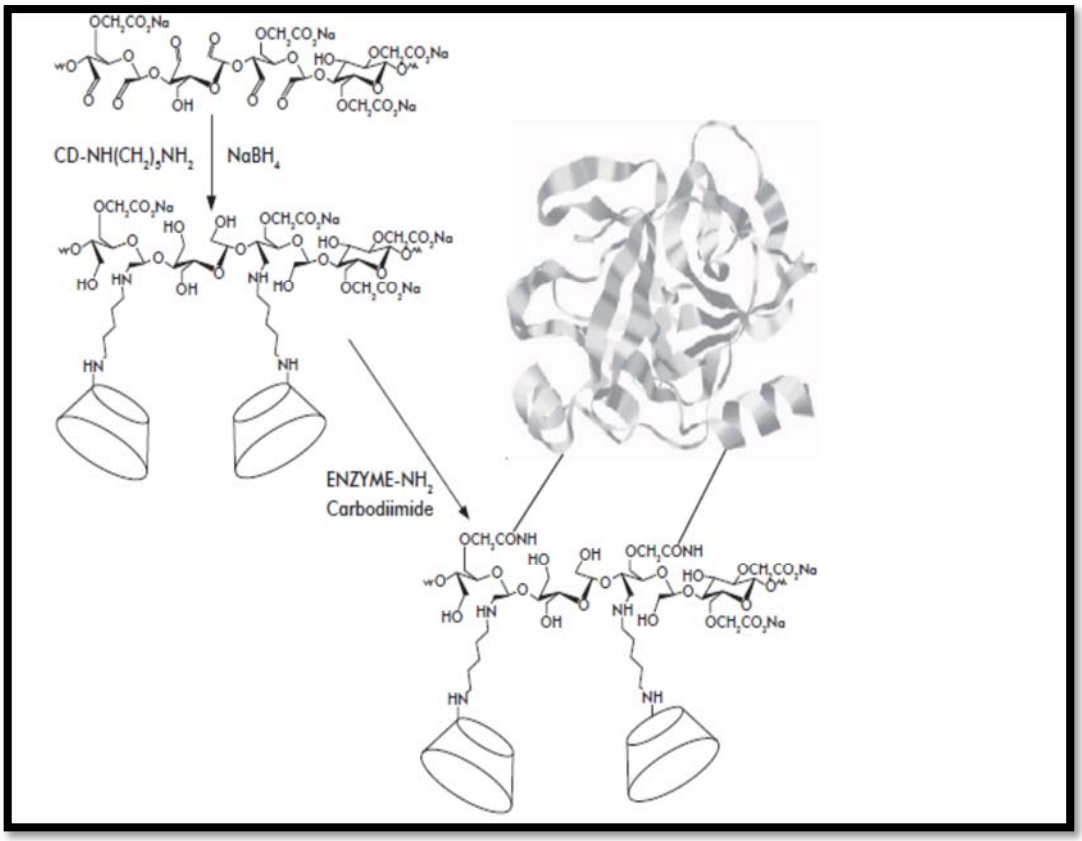


Figure 2.18 Synthesis of neoglyco enzymes with CD-branched Carboxymethylcellulose polymer.

This polysaccharide changed into covalently related to free amino companies located on the floor of GOx by using a water-soluble carbodiimide as coupling agent. The carbohydrate content of the modified enzyme become predicted to be 244 mol.

Affording to the chemical shape of "CMC-CD and the sugar content of the local enzyme, this result represents a mean of 0.78 mol of CMC attached to each mol of GOx. The enzyme retained 67% of its unique interest after conjugation with CMC-CD". An average of 21 mol of amino corporations changed into observed for the GOx-CMC-CD conjugate, as envisioned with the o-phtaldehyde technique [116], [117].

In this complex, 27 moles of amino companies in step with mol of protein were modified through the equal polysaccharide molecule, factor toward that the conjugate has a high degree of intermolecular crosslinking. Native and changed paperwork produced "km values of 0.06 µmol/l and 1.09 µmol/l, respectively. glycosidation decreased the affinity of GOx ford glucose by18-fold. Values of Kcat/km for the local and changed enzymes were 11.33 µmol/l•s and 1.31 µmol/l•s respectively".

As noted above, poorer catalytic overall performance of the changed enzyme can be for the reason that of steric quandary from the connected polymer chains, causing in lower accessibility of the energetic web site for the substrate. Further, “the localized electrostatic gradient can also have an effect on km through facilitating or obstructing interactions between charged businesses within the enzyme or enzyme-substrate complex all through binding and catalysis. massive variety of small lively gains and losses may complicate the analysis of such average outcomes” [118].

Similar results on catalytic pastime were formerly described for different enzyme polysaccharide conjugates and for GOx entrapped in liposomes. Importantly, hydrophobic interactions among cd and hydrophobic amino acid residues might also lessen the dielectric constant of the enzymatic surroundings. Such a phenomenon might change the dissociation constants of acid and fundamental companies in enzyme substrate and buffer.

Partition might motive modifications in nearby concentrations of those molecules, affecting in turn the apparent kinetic parameters of the enzyme. “Covalent glycosidation of glucose oxidase with cd-branched carboxymethylcellulose polymer constitutes therefore an effective method for boosting the steadiness of this enzyme” [119], [120].

The multipoint interactions present multimeric enzymes may additionally save you subunit dissociation via intersubunit linking at the same time as concurrently decreasing conformational inactivation. The better thermal balance of GOx after glycosidation the ”CMC-CD is crucial result [121].

Thermal denaturation of GOx proceeds specifically thru the destabilization of ionic and hydrophobic interactions and the breakage of hydrogen bonds and van der Waals forces, leading to conformational modifications in tertiary structure inactivate the enzyme. The conformational balance located for GOx modified with CD polymers results from the cooperative contribution of several factors, which include the covalent cross-linking to macromolecular structures and the formation of recent hydrogen bonds and electrostatic interactions at its surface .The incidence of host visitor interactions between the connected CD moieties and the amino acid residues at the surface of the changed proteins are also related to the thermal stabilization [122], [123].

Further, the changed enzyme become stronger after incubation in answers at exceptional pH values Premiere pH become 6.0 for both local and CMC changed GOx, as reported

earlier. However, CMC-cd-modified GOx retained 70% of its activity at pH 7.5 in comparison with 20% for the native enzyme. The truth that “the polymer-enzyme complex became more solid at this pH than its native. It suggests that the connected polymer chain then conserve lively enzyme conformation at alkaline pH and avoids protein unfolding”.

The “CMC-CD derivatization would conformational stabilization may have its basis on the high degree of intermolecular cross-linking of the conjugate, due to covalent multipoint attachment of the polymer chains to the enzyme protein shape” [124].

2.5.4 Invertase stabilization by chemical modification of sugar chains with carboxymethylcellulose

Enzymes that “action in homogeneous systems are required for numerous biomedical commercial and biotechnological applications. Among the various approaches described for stabilizing Enzymes in aqueous media is chemical modification using water-soluble macromolecules. The degree of amino institution modification of the CMC-Invertase conjugate decided by the colorimetric quantification of free amino activities in the ethylenediamine activated enzyme earlier than and after conjugation with CMC changed into predicted as 63%”. In keeping with this result, a median of 31 amino corporations had been involved inside the conjugation process [125].

the extra carbohydrate content due to the CMC of the polymer-changed enzyme changed into calculated as 526 mol of monosaccharide (as glucose) per mol of invertase, considering the preliminary sugar content in the glycoenzyme invertase 1810 according to mol of protein decided by means of the phenol-sulfuric acid technique [126].

The result represents a median of 3.5 CMC connected to every mol of holoenzyme. Taking into consideration the polysaccharide content material as well as the modification fee of amino corporations determined for the CMC-invertase complicated immoderate degree of intermolecular cross-linking became anticipated for this conjugate. In reality, a mean of nine mol of amino organizations have been changed by using one mol of polymer for every mol of adduct. The specific activity of the polymer-conjugated enzyme was 1030 Umg-1 of protein, which represents about 56% of the initial invertase activity.

Overall, “a decline in activity is observed for enzymes immobilized on macromolecular supports due to steric hindrance by the polymeric structure, which can mask the enzyme activity site from interacting with the substrate” [127].

Changed enzyme become significantly more solid at temperatures higher than 60oC in assessment with native invertase. The value T 50oC, defined because the temperature at which 50% of the preliminary changed into retained, increased from 64oC for the local enzyme to 70oC, for the invertase – CMC conjugate. The kinetic facts for the thermal inactivation of local and CMC changed enzyme uncovered at a diffusion of temperatures.

At some stage in “1 h of incubation, both invertase arrangements lost interest gradually with time in keeping with a first-order kinetic however the inactivation charge constants (ki) for the changed enzyme were continually decrease than that similar to native counterpart. Consequently, the half of life (t1/2) for invertase at each temperature were markedly improved after conjugation with CMC [128].

This effect was especially stabilization for CMC-modified invertase. It is well known that enzymes display improved thermal stability after covalent modification with water-soluble polymers by multipoint attachment. This behavior was attributed to the reduction of the protein mobility after conjugation

Immoderate degree of covalent intermolecular cross-linking have become anticipated for the CMC invertase conjugate. In addition, the polyanionic nature of CMC favors the formation of ionic interactions with certainly charged corporations on the surface of invertase. Certainly intermolecular salt bridges are one of the maximum critical forces contributing to the safety of enzymatic active conformations [130]. Although “the energetic contribution of surface salt bridges to protein stabilization is much less effective than these corresponding to suppressed ion couples, in the study the high degree of carboxymethylation shown by CMC could favor the formation of multiple ion interactions at the surface of modified invertase” [131].

In this regard, “the total energy contribution of these interactions would be important to the conformational stability of the invertase, increasing the degree of cross-linking in the complex by multipoint surface salt bridges

This hypothesis is supported by the lower stabilization shown by the conjugated enzymes after incubation at extreme acidic conditions. Under these conditions, all the CMC carboxylate groups were renewed into the acid form consequently and all the possible stabilizing ion-pair interactions between the protein and the polymer were disrupted".

The covalent and ionic cross-linking also contributed to save you dissociation processes inside the changed enzyme exposed to thermal remedy. [132].

the effect of "pH on enzyme stability was evaluated by using incubating local and modified invertase at 30ºC at one-of-a-kind pH values for 30 min. underneath those conditions, the pH balance improved for the CMC changed invertase in the pH variety evaluated".

At alkaline pH, values the improved balance for CMC-invertase complicated become better than for acid ph. in truth, multiplied balance of approximately 60 and 30% turned into received for polymer-invertase complicated after incubation at pH values of 12.0 and a pair of 2.0, respectively. In assessment with the previous consequences, the attachment of CMC to invertase constitutes a handiest device for enhancing pH balance to invertase.[133].

2.5.5 Stabilization and Functional Properties of Escherichia coli Penicillin G Acylase by Conjugation of Anionic Polysaccharide Carboxymethylcellulose

Stabilization of escherichia coli penicillin Gacylase (PGA) conjugated with carboxymethylcellulose (CMC) against temperature and pH became studied. The 2,3-dialdehyde derivative of CMC obtained by way of periodate oxidation turned into covalently conjugated to PGA by Schiff's base formation.

The inactivation mechanism of each native and CMC-conjugated PGA appeared to obey first order inactivation kinetics for the duration of prolonged incubations at 40ºC–60°C and on the pH variety 4–9. Inactivation price constants of conjugated enzyme were constantly decrease, and half of lifestyles times were continually higher than that of local PGA [134].

Protein content material of enzyme solutions become decided via the blue binding technique the use of bovine serum albumin (BSA) as preferred.

The hydroxylamine technique was used to modify enzyme activity all through purification method. Because of its higher sensitivity and rapidity, the p-dimethyl amino benzyl dehyde (PDAB) technique become used for kinetic investigations [135].

One unit of enzyme interest is defined as “the amount of enzyme required to produce 1 μmol of 6-APA per min at 40oC and pH 8.0 from 15 mM pen G in 50 mM phosphate buffer”. the values unfastened electricity of inactivation (ΔGi) of CMC-conjugated enzyme were observed to be constantly better than that of local PGA in any respect temperatures and pH values studied as another indicator of enzyme stabilization. Highest stability of CMC-conjugated enzyme turned into determined as almost four-fold at 400C and PH 8.0. adjustments were determined on the temperature and pH profiles of PGA after CMC conjugation. Decrease km and better kcat values of PGA obtained after CMC conjugation shows the stepped forward effect of conjugation at the substrate affinity and catalytic performance of the enzyme [136].

CHAPTER 3

MATERIALS AND METHODS

Carboxymethylcellulose (CMC) sodium salt (90 kDa Mw, substitution degree= 0.7, degree of polymerization=196), 1-Ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC) (molecular formula: C8H17N3·HCl, MW=191.7, activator of carboxylate groups of CMC), alkaline phosphatase (ALP) and p-nitro phenyl phosphate (PNPP) (substrate of ALP) were obtained from Sigma-Aldrich (Table 2). Phosphate buffer saline (PBS), it is a water-based salt solution the system include the following reagent: 0.22g/L sodium hydrogen phosphate (NaH2PO4), 1.82g/L sodium hydrogen phosphate (Na2HPO4), 8.76 g/L sodium chloride (NaCl), pH 7.2.

Table 3.1 Used chemicals

Chemical	Source
Carboxymethylcellulose (CMC)	Sigma
p-Nitro phenyl phosphate (PNPP)	Sigma
Alkaline Phosphatase (ALP)	Sigma
Na_2HPO_4 , NaH_2PO_4 , Glycine	Sigma
KOH	Merck
HCI	Riedel-de Halen
$ZnCl_2$, $MgCl_2$	Merck
1-Ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC)	Sigma
NaCl	Fluke

3 .1.2 Preparation of Solutions

3.1.2.1 Substrate Buffer

1g of glycine (C2H5NO2, Mw = 75.07 g /mol) was dissolved in 50 mL of distilled water and then adjusted to pH = 10.4. Then 0.027g of Zinc chloride ($ZnCl_2$, Mw = 136.29 g/mol) was weighed and dissolved in 50 ml of distilled water. 0.040 g Magnesium chloride ($MgCl_2$, Mw = 95.3 g / mol) were weighed and dissolved in 50 mL of distilled water. These three solutions were then mixed and total volume was completed to 200mL by adding distilled water. It was stored +40C

3.1.2.2 Substrate (PNPP) Solution

25 mg of p-nitro phenyl phosphatase (C6H6NO6P2C4H11NO3 , Mw = 461.4 g /moI) was dissolved in 25 mL of substrate buffer to give 1 mg substrate / 1 mL substrate solution. The substrate solution was stored in the dark.

3.2 Determination of Alkaline Phosphatase Activity

1.2 mg of alkaline phosphatase (ALP) was prepared by dissolved in 1.2 mL of phosphate buffer saline (PBS) pH = 7.2 and stored at +4oC. Enzyme activity in amounts spread out from 80-800 ng was determined in the presence of PNPP. For this purpose, the PNPP solution was added to the wells and the color absorbance at 405 nm was measured in the ELISA reader after one hour of waited in the dark. Each experiment was performed in three replicates.

3.3 Preparation of Alkaline Phosphatase-Carboxymethylcellulose Conjugates

For the preparation of enzyme-particle conjugates; six reactions were formed .The volume of each reaction was prepared to be 30 m. After pH of 1-Ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC) in dry state was in tune to 4.8, EDC was added to the reactions and the polymers (CMC) were activated. After stirred for 2 hours at +4oC, the enzyme (ALP) was added drop wise to these reactions. Mixing was carried out at +4^{o}C overnight.

Table 3.2 Preparation of reactions of ALP-CMC conjugates.

The reaction Chemical	CMC (mg)	EDC (mg)	ALP (µg)
1.reaction	0	0	30
2. reaction	15	45	30
3. reaction	30	90	30
4. reaction	60	180	30
5. reaction	90	270	30
6. reaction	150	450	30
PBS	0	0	0

3.4 Determination of pH Stability of Enzyme and Conjugates

After termination of the reactions, each reaction was divided into 3 portions and then the pH values were adjusted to pH = 5, pH = 6 and pH = 7 with appropriate buffers .

Using alkaline phosphatase (ALP) substrate PNPP, 30 µl of each sample was taken to check for the activities.

3.5 Determination of Thermal Activity of Enzyme and Conjugates

In order to determine the thermal stability of ALP enzyme and conjugates, reactions were treated at 50ºC, 60ºC, 70ºC and 80oC for 5 min. After incubation, immediately, using PNPP substrate solution, 30 µl of all sample was taken to check for the activities.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of Alkaline Phosphatase Activity

After the preparation of the enzyme, the activity of the diluted enzyme in the appropriate amounts was determined by using PNPP. Since the absorbance of 800 ng of enzyme at 405 nm is high, lower value from 2.5 was used. The enzyme was decided to be used in conjugation reactions (Figure 19).

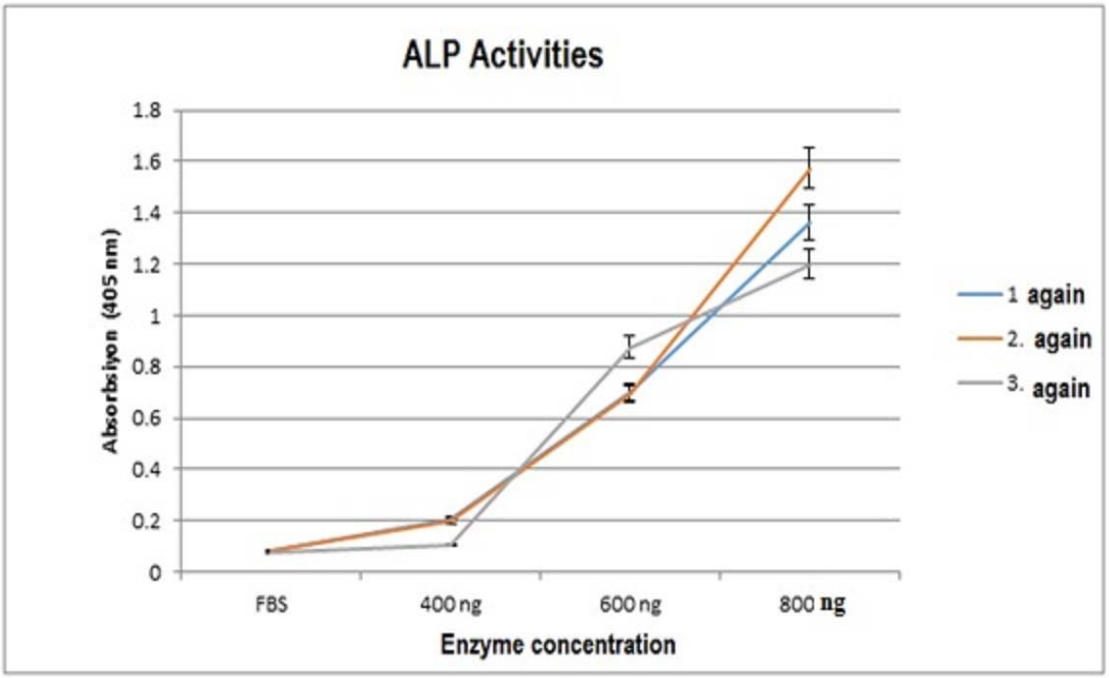


Figure 4.19 Results of ALP enzyme activity

4.2 Preparation of ALP and Carboxymethylcellulose Conjugates

30 µg of the enzyme was used for each conjugation reaction having 30 ml reaction solution. Each reaction was carried out as labelled. As it is seen from, the concentrations of both CMC and EDC were increased while concentration of ALP was constant. Total six different conjugates were prepared.

4.3 pH Effect on Stability of Enzyme and Conjugates

The activity changes of the enzyme and the conjugates after pH adjustment were calculated from the spectral measurements at 405 nm (Figure 20). It was found that all reactions except reaction 1 (having only enzyme) showed higher stability at pH = 5 than the values at pH 6 and 7.

It was found that the conjugation increased the stability of the ALP when conjugates are compared to the free enzyme. At the same time, increasing of amount of both CMC and EDC in conjugates also increased the stability of enzyme at pH = 5. As a result of this experiment, it was determined that the stability of reaction 6 was higher than the other 5 reactions when the values of six different reactions are compared to each other at pH 5, 6 and 7, respectively. The pH stability was greater than before CMC modified ALP

This improved stability could also result from the contribution of two other possible effects: the electrostatic interactions between the cationic residues from the protein and the carboxylate groups from CMC and the formation of hydrogen bonds between hydroxyl groups from the polysaccharide backbone and hydrophilic groups from the enzyme.

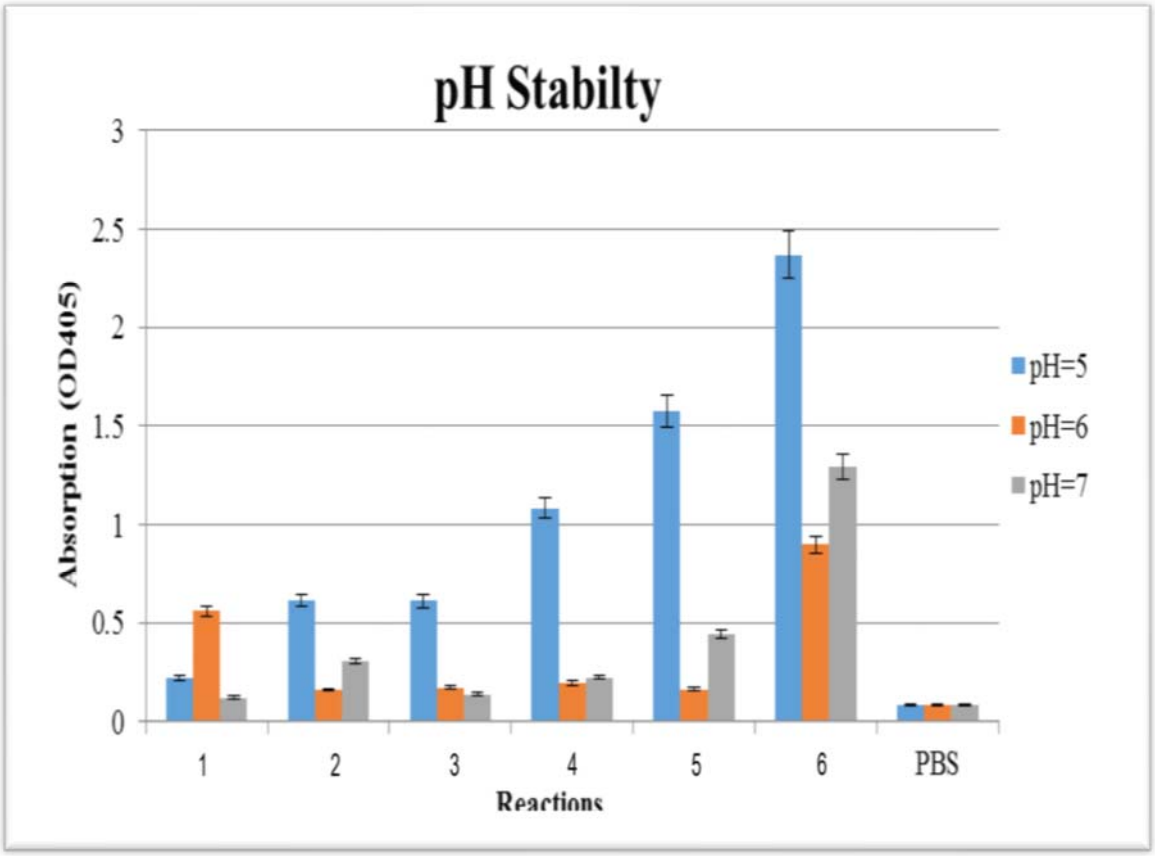


Figure 4.20. Thermal Activity of Enzyme and Conjugates

Following the pH stability assay, the thermal activities of the native enzyme and ALP-CMC conjugates were determined. Therefore, the best-defined pH stability of each reaction was selected and the thermal stability experiments were done. At thermal stability experiments, pH = 6 for reaction 1 and pH = 5 for reaction 2, 3, 4, 5 and 6 were selected because of high stability at the each reaction. While the activity of free ALP was high at 50ºC, 60ºC, the activity of free ALP was significantly reduced at 70ºC and 80ºC. The results for all other conjugate reactions were similar. When the effects of all temperatures on the all reactions are taken into considered, reaction 6 gave the highest stability followed by reaction 5, 4, 3, 2 and 1 respectively. Conjugation with CMC, especially, at reaction 6 was increased ALP resistance to both pH and temperature change (Figure 20).

4.4 Thermal Activity of Enzyme and Conjugates

Following the pH stability assay, the thermal activities of the native enzyme and ALP-CMC conjugates were determined. For this purpose, the best-defined pH stability of each reaction was selected and the thermal stability experiments were done. At thermal stability experiments, pH = 6 for reaction 1 and pH = 5 for reaction 2, 3, 4, 5 and 6 were selected because of high stability at the each reaction (Figure 4.2).

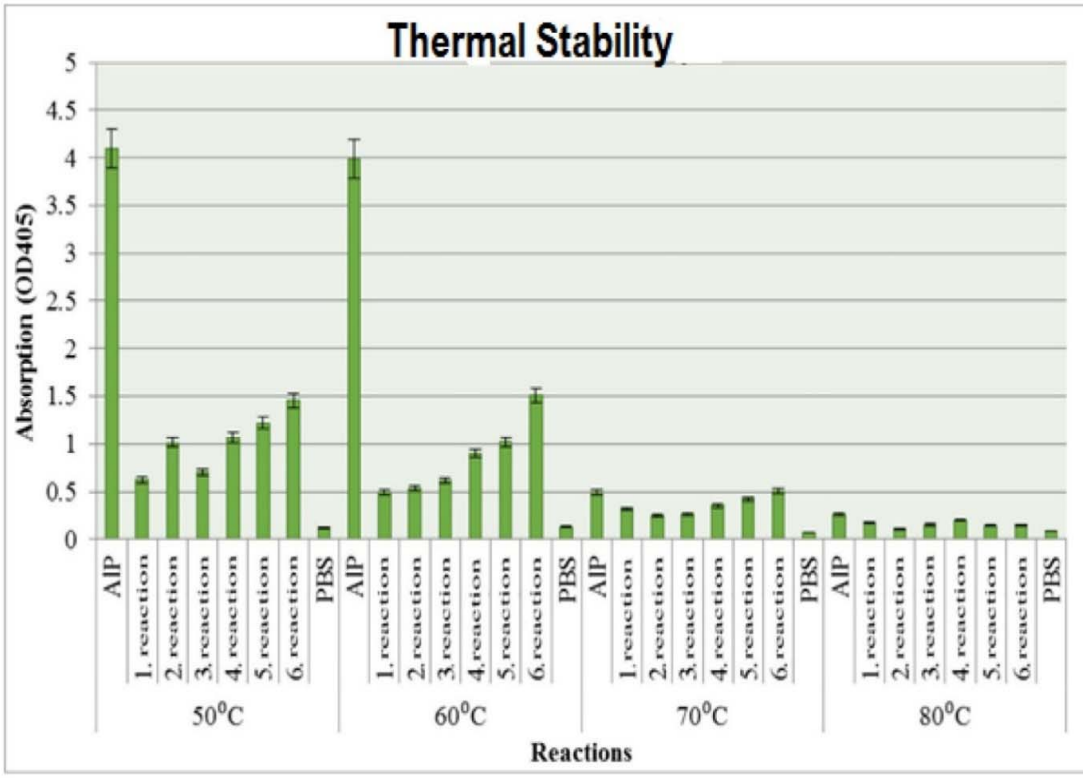


Figure 4.21 Thermal activity of ALP enzyme and conjugates (ALP: 1 µg enzyme stored in PBS with pH = 7.2 at +4ºC).

While the activity of free ALP was high at 50ºC, 60ºC, the activity of free ALP was significantly reduced at 70ºC and 80ºC (Figure 21). The results for all other conjugate reactions were similar. When the effects of all temperatures on the all reactions are taken into considered, reaction 6 gave the highest stability followed by reaction 5, 4, 3, 2 and 1 respectively. Conjugation with CMC, especially, at reaction 6 was increased ALP resistance to both pH and temperature change.

4.5 Discussion

Carboxymethylcellulose (CMC) was activated with l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). After activation, 2.3-dialdehyde derivative of CMC changed into covalently conjugated with alkaline phosphatase (ALP) by using Schiff's base formation among the aldehyde companies on CMC and unfastened amino groups at the ALP molecule as a way to reap CMC-ALP complicated. The pH consequences of CMC-ALP complex treated underneath extraordinary concentrations. The stabilities of CMC-ALP complex had been expanded at pH 5 and 7 in reaction 2, 3, 4 and five when they as compared to response 1, which has only local enzyme. The CMC-ALP complex was substantially the most stable at pH 5, 6 and 7 in response 6 due to excessive attention of CMC and EDC in Figure 20

The CMC-ALP complex changed into investigated by way of heating the samples for 10 min at temperatures starting from 50ºC to 80ºC. The thermal stabilities of the CMC-ALP complex were exceptionally lively at 50°C and 60°C in reaction 2, 3, four, five and six due to high awareness of CMC and EDC as visible in Figure 21 after they as compared to response 1 which has simplest local enzyme. 50°C and 60°C suggest that multipoint attachment of polysaccharides to the enzyme surface increases their thermo stability [122].

The conjugation become accompanied by decrease inside the unique interest of ALP enzyme after temperatures multiplied at 70oC to 80oC. In popular, a lower in interest is observed for enzymes immobilized on macromolecular supports because of steric challenge via the polymeric shape, which can mask the enzyme activity site from interacting with the substrate [122].

Five studies were located associated with conjugation with CMC of enzymes so that you can boom stability of enzymes. by way of comparing consequences to preceding look at of conjugation between CMC and different enzymes which includes trypsin the preliminary hobby changed into multiplied from 55ºC to 62ºC, in assessment to the native protease, as well, the pH balance was expanded for the modified trypsin inside the range of pH between 2.0 and 6.0. At decrease values of pH, the enhanced stability was large than at alkaline pH due to the fact the presence of a lysine price at area 188 appearing an important function in regulating the pH variety of catalysis for trypsin; the changed trypsin retained 62% of the original esterlytic interest [137].

The specific interest of the modified enzyme toward casein was 42% of the native enzyme, closer to a small substrate is benzoylarginineethylester (BAEE). The covalent attachment of CMC to the surface of trypsin effects in some changes within the structural and functional properties of the enzyme. This transformation may want to contribute to accelerated most appropriate pH range [138].

The conjugate become also more proof against the motion of denaturant sellers such as urea and sodium dodecyl sulfate [139]. The polysaccharide CMC used as editing agent for glucose oxidase (GOx) changed into prepared through oxidizing CMC with sodium metaperiodate, accompanied by using conjugation to mono-6-hexylenediamino-6-deoxy-ß cyclodextrin inside the presence of nabh4. The conjugation of CMC with cd moieties yielded a water-soluble macromolecular shape with a 28:1 cd-polymer ratio [140].

This polysaccharide became covalently connected to free amino corporations located at the surface of (GOx) the usage of a water-soluble carbodiimide as coupling agent as visible in .the thermal balance of the changed enzyme turned into investigated by means of heating the samples for 10 min at temperatures starting from 25ºC to 65ºC.

Modified GOx became extra proof against temperatures above 30ºC than the native enzyme, as T50 extended from 45ºC before glycosidation to 51ºC afterwards. Comparable results have been mentioned for GOx stabilized thru the addition of KCl and for exclusive GOx immobilization variations [141]. Invertase from saccharomyces cerevisiae modified into activated by periodate treatment and in addition reacted with ethylenediamine sodium borohydride

The modified enzyme contained 3.5 mol of polysaccharide in step with mol of holoenzyme, and retained approximately 56% of the initial invertase interest. The thermo stability of invertase accelerated from 64ºC to 70ºC become certainly decreased for Polymer-changed enzyme. Growth of 9.1kJ mol^{-1} in activation loose strength of inactivation changed into decided for invertase after change. Purposeful stability become expanded for CMC-invertase complex inside the range of pH among 2.0 and 12.0. The conjugate became also extra proof against denaturation with the aid of 6M urea solution.

Beneath those conditions, the pH balance expanded for the CMC changed invertase inside the pH range evaluated. The improved balance for CMC-invertase complex

changed into better than for acid pH. In fact, an accelerated balance of approximately 60 and 30% was obtained for polymer invertase complex after incubation at pH values of the attachment of CMC to invertase 2.0, respectively. In comparison with their preceding consequences constitutes only device for enhancing pH stability to invertase.

The stabilization of Escherichia coli penicillin G acylase (PGA) conjugated with CMC in opposition to temperature and pH turned into studied. The 2,3-dialdehyde by-product of CMC acquired through per iodate oxidation changed into covalently conjugated by Schiff's base.

Formation. The activation mechanism of each local and CMC-conjugated PGA regarded to obey first order activation kinetics throughout extended incubations at 40–60oC and inside the pH range 4–9. Inactivation charge constants of conjugated enzyme had been always lower, and 1/2 existence times were constantly better than that of native PGA.

The activation loose power of activation (ΔGi values) of CMC conjugated enzyme was observed to be continually higher than that of local PGA the least bit temperatures and pH values studied as a few different indicator of enzyme stabilization. Maximum stability of CMC-conjugated enzyme have become located as nearly 4-fold at 40oC and pH 8.0. Decrease km and higher cat values of PGA received after CMC conjugation suggests the advanced impact of conjugation on the substrate affinity and catalytic performance of the enzyme [142].

In comparison with previous results at different enzymes with different optimum pH values, the pH value at which the bonds with are influenced by H+ in such a way that the shape of their active site is the most complementary to the shape of their substrate. The high temperature can break the enzyme. The enzyme can be denatured and can have no longer function.

In conclusion, the stabilization of alkaline phosphatase by covalent conjugation of CMC could be explained by the effect of several factors on the native conformation of enzyme: (a) The rise of the inflexibility of active enzyme conformation due to formation of intermolecular cross-links for the period of the multipoint covalent attachment of polymer chain to the enzyme molecule (b) formation of intermolecular salt viaduct by electrostatic contacts between the negatively charged carboxylate groups of CMC and positively charged groups of ALP molecules, (c) hydrogen bond

formation between the hydrophilic collection ALP charged carboxylate groups of CMC The results reported in the articles suggest that covalent modification of enzymes by the anionic polysaccharide CMC might be a new and advantageous tool for improving enzyme stability under various considering the advantages of alkaline phosphatase and its use in different biomedical, industrial and biotechnological applications

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