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Stabilization of *Bacillus Licheniformis* ATCC 21415 Alkaline Protease by Immobilization and Modification

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Abstract: Alkaline protease from *Bacillus licheniformis* ATCC21415 was partially purified by fractional precipitation at 70% ethanol which showed 6.2-fold purification. Immobilization of the enzyme by physical adsorption on loofa (as a new carrier) had the highest immobilization yield (70.5%).Chemical modification of the enzyme by covalent coupling with sodium -periodate activated amylopectin retained (78.3%) of the original activity. Immobilized and modified enzymes retained 59.9 and 76.1%, respectively of the original activity after heating at 60^{o}C for 60 min while the native enzyme retained 19.4%. The calculated half-life values ($t_{1/2}$) of heat inactivation at 60^{o}C for modified, immobilized and native protease were 115 , 100and 25 min, respectively. Activation energy (E_A) of the native enzyme was 23.6Kcal/mol which higher than those of immobilized and modified enzymes (18.9 and 19.9Kcal/mol, respectively). The immobilized and modified forms exhibited lower V_{max} and higher K_m values compared to that of the native form. Immobilized and modified forms were more stable in presence of EDTA than the native form. The crude enzyme digested some natural proteins and was able to extract collagen from chicken skin.

Keywords: *B. licheniformis* ATCC 21415, alkaline protease, immobilization, modification

INTRODUCTION

Proteases execute a large variety of functions and have important biotechnological applications. They represent one of the three largest groups of industrial enzymes and find applications in detergents, leather, food, pharmaceutical industries and bioremediation processes (Gupta *et al*., 2002). Probably the largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing (Banerjee *et al*., 1999). The enzyme should be stable and active in the presence of typical detergent ingredients for use in detergent (Najafi *et al*., 2005). Stabilization of enzymes has received much attention in recent years. Stabilization against thermal inactivation can be performed in several ways such as cross-linking to a water insoluble carrier with a bifunctional reagent or covalent coupling to natural and synthetic polymers and entrapment in gels (Najafi *et al*., 2005). Chemical modification with low molecular weight monofunctionl reagents cross-linking with bifunctional reagents polymer attachment has been reported by Ben Ammar *et al*. (2002) and Fernandez *et al*. (2002). Many proteins containing carbohydrate residues exhibit increased thermal stability towards heat and storage, which in many cases seems to be due to the carbohydrate part of the molecule. Most glycoproteins exhibit high water solubility, and thus it was considered promising to be stabilized water-soluble enzymes through covalent attachment to carbohydrates (Abdel-Naby 1999). The mechanism involved in the carbohydrate-induced stability of glycosylated proteins by rigidification of the conformation was reported by Klibanov (1983). On the other hand, Srivastava (1991) argued that the hydration effect of the attached carbohydrate may be responsible for improving the stability of conjugated enzymes. Hydrogen bonding between the polysaccharide and protein surface has been suggested as causes of thermal stabilization of the synthetic glycoproteins (Lendewrs *et al*., 1984). For industrial applications, the immobilization of protease on a solid support can offer several advantages, including repeated usage of enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bed reactors (Abdel-Naby *et al*., 1998). Enzyme stabilization will thus continue to be a key issue in biotechnology. The present study deals with partial purification of *B. licheniformis* ATCC 21415 alkaline protease and its stabilization by immobilization and chemical modification. The catalytic properties and the stability of the immobilized and modified enzymes were compared to those of the native enzyme.

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MATERIALS AND METHODS

Strain:

Bacillus licheniformis ATCC 21415 was obtained from the American Type Culture Collection, USA.

Production Media:

The culture medium for enzyme production was defined as follows (g/l): KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 1.0; $CaCO_3$, 1.0; NaCl, 1.0; dextrin, 35; peptone, 1.0; $(NH_4)_2SO_4$, 1.0 The components were dissolved in wheat bran extract (5%) and pH 7.0. The Erlenmeyer flasks (250) containing 25ml of sterile medium. The flasks were inoculated and then incubated at 37°C for 96h in a rotary shaker regulated at 180 rpm. The cells harvested by centrifugation at 5000 g for 20min in a refrigerated centrifuge at 4°C.

Protein Assay:

Protein was measured by the method of Lowry *et al*. (1951) with bovine serum albumin (BSA) as a standard.

Enzyme Assay:

Protease activity was determined as reported earlier Bergkvist (1963) by determination of the amount of casein hydrolyzed. One unit (U) of enzyme activity corresponded to the amount of enzyme which liberated one µmol tyrosine per min.

Thermal Stability:

Thermal stability of free, immobilized and modified protease were tested by incubating the enzymes in glycine-NaOH buffer (0.1M, pH 9.0) at a designated temperature (30 -90min) before activity assay.

pH Stability:

The enzyme was incubated at 30°C in 0.1M glycine-NaOH buffer of different pH values (pH 6-11) for 60min.

Digestion of Natural Proteins:

The crude enzyme (10U) was incubated with human blood clot, coagulated egg white and chicken skin in glycine-NaOH buffer (0.1M, pH 9.5) at 35°C /10h.

Alkaline Protease Modification:

The modification was carried out according to Ben Ammar (2002).

Oxidation of Polysaccharides:

250 mg of each polysaccharide were dissolved into 10ml of 0.1M sodium periodate solution and allowed to stand at 30°C for 6h. After that, 0.3ml of ethylene glycol were added and allowed to react for 1h. The reaction mixture was dialyzed against water at 4°C overnight, and then lyophilized.

Enzyme Coupling with Activated Polysaccharides:

0.4mg of alkaline protease and 100mg of oxidized polysaccharides were combined in glycine-NaOH buffer (0.05M, pH 9.0). The conjugates were precipitated at 50% ethanol and lyophilized.

Immobilization Methods:

Physical Adsorption:

One gram of the carriers were incubated with 1ml of enzyme solution (220U) at 4°C overnight. (Abdel-Naby *et al*., 1998).

Covalent Binding:

One gram of the carriers were treated with 2ml of 2.5% (v/v) glutaraldehyde for 2h at 30°C. Then washed with distilled water to remove the excess glutaraldehyde. The wet carriers were mixed with 1ml of enzyme solution (220U) and incubated for 6h at 30°C. (Abdel-Naby *et al*., 1998).

Ionic Binding:

0.5gm of the cation or anion exchanger was equilibrated with acetate buffer (0.1M, pH 6.0) or Tris-HC buffer (0.1M, pH 9.0). The carriers were incubated with 1ml of enzyme solution (100U) at 4°C for 12h. (Abdel-Naby *et al*., 1998).

Entrapment:

In Agar:

10ml of different concentration of agar solution (2, 4, 6%) at 45°C were mixed with enzyme solution(220U). The mixture was quickly cooled to 4°C, cut into 1x1x1cm^3 fragments. (Cheetham *et al.*, 1985).

In Ca-alginate:

100ml of different concentrations of sodium alginate solution (2, 4, 6%) were mixed with enzyme solution(220U). The entrapment was carried out by dropping the alginate solution in 0.1M $CaCl_2$. The resulting beads (0.5-1.0mm diameter) were collected. (Abdel-Naby 1993).

RESULTS AND DISCUSSIONS

The results of a typical procedure for the partial purification of *B. licheniformis* ATCC21415 alkaline protease are summarized in Table (1) pointed to the most active fractions produced by ethanol, acetone and ammonium sulphate. The highest specific activity was achieved by precipitation at 70% ethanol (16.4U/mg protein) and showed 6.2-fold purification. This fraction was used for the succeeding part of work.

Ammonium sulphate at 60 and 70% saturation recovered 14.39 and 13.73% of initial activity with 3.6 and 3.9-fold purification. *Pseudomonas aeruginosa* protease showed 3.6-fold purification by using ammonium sulphate 70% (Najafi *et al*., 2005). The lowest recovered activity obtained at 50% acetone (1.4-fold purification), this result was similar to that obtained by Thangam and Rajkumar (2002).

Table 1: Partial purification of *B. licheniformis ATCC21415* alkaline protease.

Purification	Total protein (mg/fraction)	Total activities(U)	Recovered activity (%)	Specific activity (U/mg protein)	Purification (-fold)
Crude Enzyme	500.0	1433.0	100.0	2.9	1.0
Ethanol 70%	20.3	331.9	23.2	16.4	6.2
Acetone 50%	23.4	96.8	6.8	4.1	1.4
$(NH_4)_2SO_4$					
60%	19.9	206.2	14.4	10.4	3.6
70%	17.8	196.7	13.7	11.1	3.9

Ethanol fraction precipitated at 70% concentration was used for enzyme immobilization. Enzyme immobilization by physical adsorption (Figure 1) indicated that the lowest immobilization yield (36.4%) was found with chitosan. The highest yield was obtained by adsorption on loofa (70.5%) with highest immobilized enzyme (81.4U/g carrier). It was therefore selected in the following experiments and its properties were compared with those of the free enzyme.

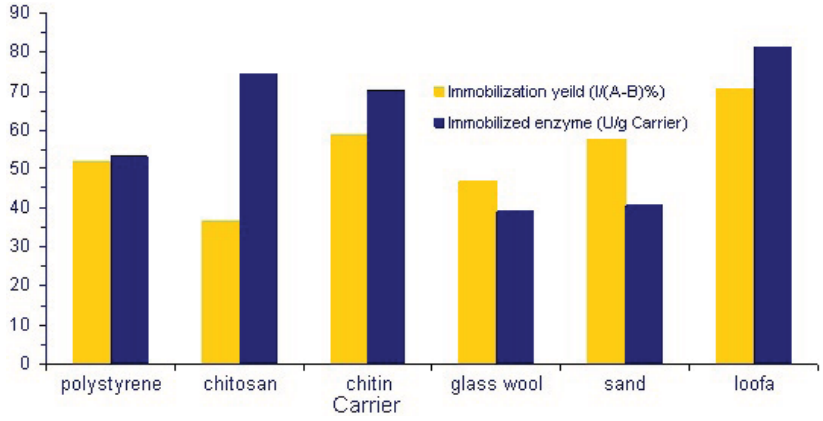

Fig. 1: Immobilization of B. licheniformis ATCC21415 alkaline protease by physical adsorption

Enzyme immobilization by covalent binding through a spacer group (glutaraldehyde) showed considerable bound (good loading efficiency) and immobilization yield (Figure 2). This good loading efficiency for the immobilization by covalent binding might have been due to the formation of stable cross linking between the carrier and the enzyme through a spacer group. In addition, covalent binding through a spacer group probably increased the local surface area of the carrier and consequently reduced the steric hindrance around the active site of the enzyme molecule Siso *et al*. (1990). The enzymes covalently bound to ceramic showed the highest immobilization yield (60.9%).

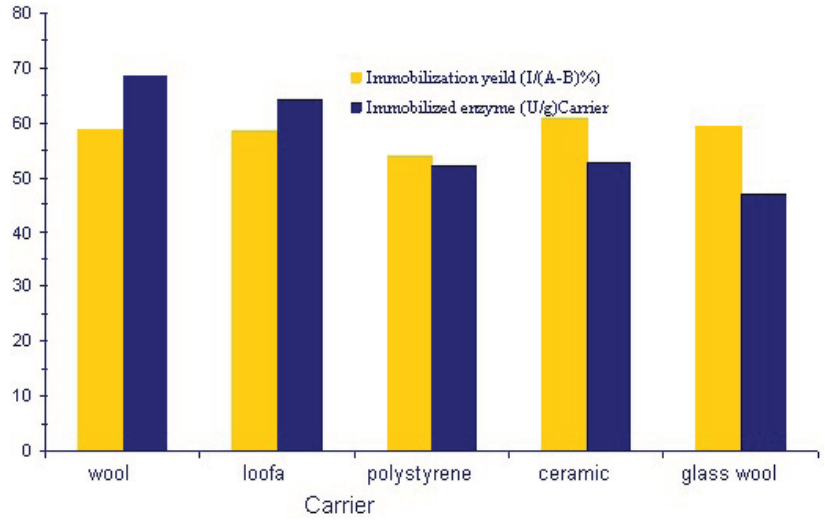

Fig. 2: Immobilization of B. licheniformis ATCC21415 alkaline protease by covalent binding.

Some ion exchangers were used for the immobilization by ionic binding (Figure 3). Ionic binding showed lower enzyme binding compared to other immobilization methods. DEAE-Cellulose DE-52 was the most suitable ion exchanger for enzyme immobilization which gave the highest activity (25.4U/g carrier) with the highest immobilization yield (44.6%). Immobilization of *Bacillus mycoides* alkaline protease by ionic binding using Amberlite IR-120 gave 28.1%U/g carrier with immobilization yield of 15% Abdel-Naby *et al*. (1998). Entrapment of enzyme in agar and Ca-alginate with different concentrations were examined. The results in figure 4 indicated that agar (4%) was sufficient for reaching maximal bound enzyme (68.9U/10mlgel) and

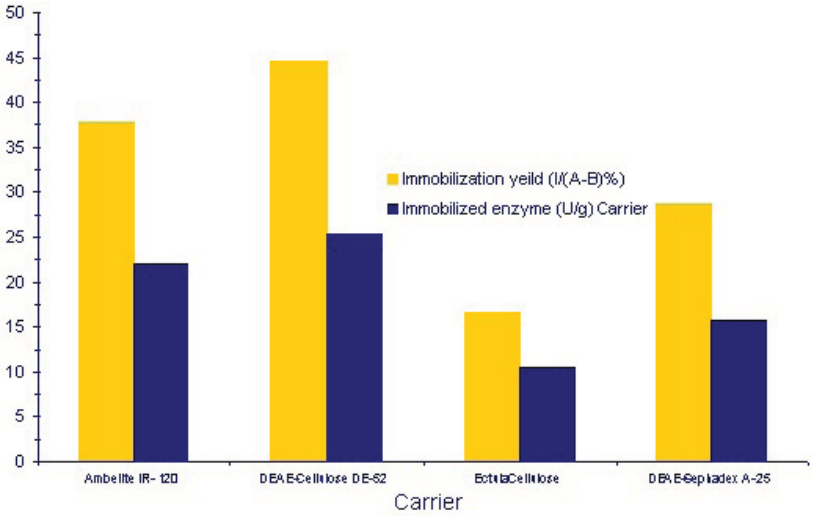

Fig. 3: Immobilization of B. licheniformis ATCC21415 alkaline protease by ionic binding.

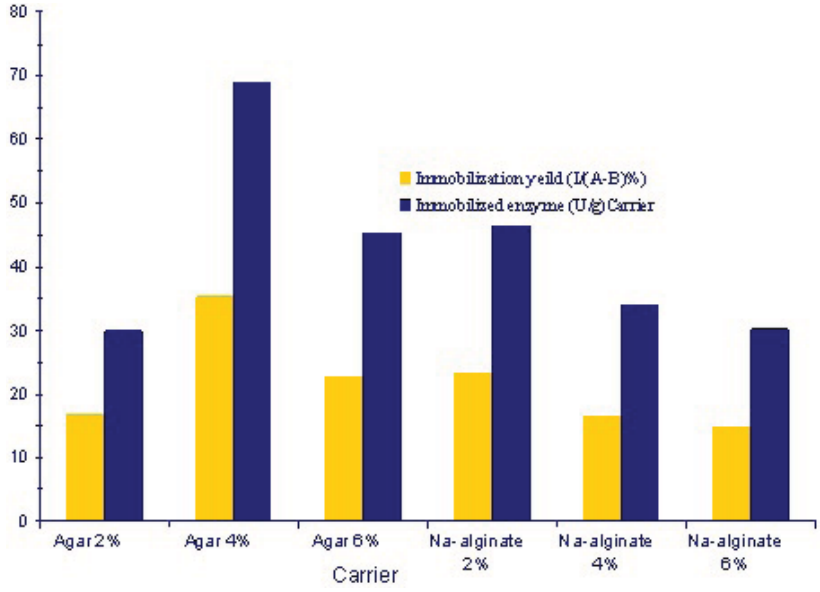

Fig. 4: Immobilization of B. licheniformis ATCC21415 alkaline protease by entrapment.

immobilization yield (35.2%). Sodium alginate (2%) had the highest immobilization yield (23.2%). The decrease in yield with increase in carrier concentration might be due to the decrease in porosity of the gel matrix which caused diffusion limitation of the substrate Siso *et al.* (1990).

Stabilization of enzyme by chemical modification was reported in Table (2). Covalent coupling of enzymes to soluble polysaccharides has been reported as a common technique for improving their properties especially thermal stability for enzyme technology Farooqi *et al.* (1997). Among all the preparations tested, the enzyme coupled to activated amylopectin showed the highest recovered activity (78.3%) with the highest specific activity (12.8U/mg protein). This recovered activity was higher than that reported by Yamagata *et al.* (1994) when coupled an alkaline protease from *Bacillus sp*. to activated dextran (56%). In general, the specific activity of free enzyme was higher than the immobilized or modified enzyme this may be due to the rigidification of the enzyme protein conformation and decrease in the flexibility of the enzyme molecule Robertson *et al.* (1996).

Thermal stability of the immobilized and modified protease compared to the free enzyme (Table 3) showed that both immobilized and modified enzymes were more resistant to thermal inactivation.

Table 2: Covalent coupling of *B. licheniformis ATCC21415* alkaline protease to activated polysaccharides.

Activated polysaccharides	* Coupled enzyme		Specific activity of conjugates (U/mg protein)	Recovered activity (%)
	Protein (mg)	Activity (U)		
Amylopectin (10^6 : 10^7)	5.98	76.32	12.76	78.3
Pectin ($3x10^4$: 10^5)	6.15	57.0	9.27	56.9
Dextran ($4x10^4$)	5.31	35.3	6.65	40.8
Dextran ($7x10^4$)	5.6	50.0	8.93	54.8
Dextran ($2x10^5$)	6.43	70.2	10.92	66.9

* Enzyme added to one gram activated polysaccharides in 10 mg protein containing 163U.

The immobilized and modified enzymes retained 59.9 and 76.1%, respectively of the original activity after heating at 60°C for 60min, however the free enzyme retained only 19.4%. Afaq and Iqbal (2001) demonstrated that immobilized papain exhibited a marked increase in thermostability and retained 87% of its original activity after 1h incubation at 65°C while the free papain lost almost 75% of its original activity. The high stability of the immobilized enzyme could be due to the diminished autolysis of the enzyme fixed to the carrier. The

Table 3: Thermal stability of *B. licheniformis ATCC21415* alkaline protease.

Temperature (ºC)	Residual activity (%)								
	Free enzyme			Immobilized enzyme			Modified enzyme		
Time of heating (min)	50	60	70	50	60	70	50	60	70
0.0	100	100	100	100	100	100	100	100	100
30	88.0	44.9	17.5	92.5	86.1	70.2	95.0	92.3	72.1
45	86.4	25.8	1.3	90.3	70.8	39.6	92.1	88.0	41.5
60	83.1	19.4	0.0	88.7	59.9	18.2	90.4	76.1	19.6
75	81.5	9.8	0.0	86.3	50.6	2.6	87.8	63.3	3.9
90	69.2	0.0	0.0	84.7	42.8	0.0	86.0	51.2	0.0

The immobilized and modified enzymes retained 59.9 and 76.1%, respectively of the original activity after heating at 60°C for 60min, however the free enzyme retained only 19.4%. Afaq and Iqbal (2001) demonstrated that immobilized papain exhibited a marked increase in thermostability and retained 87% of its original activity after 1h incubation at 65°C while the free papain lost almost 75% of its original activity. The high stability of the immobilized enzyme could be due to the diminished autolysis of the enzyme fixed to the carrier. The second possible explanation may be related to the rigidity of the conformation of the enzyme molecules resulting from binding to the carrier Afaq and Iqbal (2001). On the other hand, polymerized sucrose was evaluated as thermo protectant additive for trypsin enzyme by modification Fernandez *et al.* (2004).

The enzyme thermostability was increased (22-fold more stable) against thermal incubation at 50°C after modification processes. The stability against thermal denaturation induced by the attachment of the highly hydrophilic oligosaccharide moieties to the protease could be mainly due to the prevention of interaction between hydrophobic clusters located on the protein surface and surrounding water molecules Fernandez *et al.* (2002). Half-life time of immobilized and modified enzymes were always higher than that corresponding to the free enzyme. At 60°C the $t_{½}$ of immobilized and modified enzymes were 100 and 115min, while it was 25min for the free enzyme. Although $t_{½}$ of immobilized, modified and free enzymes at 70°C were 25, 27.3 and 7.1min, respectively. Similarly Wehidy (2005) calculated the $t_{½}$ of immobilized, modified and free *Bacillus stearothermophilus* alkaline protease at 60°C which were 46.5, 27.9 and 7.3 min, respectively.

As shown in Table 4, immobilization and modification resulted in a noticeable stabilization of enzyme against denaturation at alkaline pH. Immobilized and modified enzymes retained 69.8 and 98.1%, respectively of its original activity when pre-incubated at pH11 as compared to 38.5% inactivation seed for free enzyme. Fernandez *et al.* (2002) found that modified α-chymotrypsin was 7-fold more stabile than the free enzyme against incubation at pH 9.0.

Table 4: pH stability of *B. licheniformis ATCC21415* alkaline protease.

	Relative activity (%)		
pH	Free enzyme	Immobilized enzyme	Modified enzyme
Control	100.0	100.0	100.0
6.0	60.0	80.3	81.2
7.0	73.4	88.4	89.8
8.0	75.6	100.0	97.6
9.0	89.1	90.6	100.0
10.0	82.4	87.0	100.0
11.0	61.5	69.8	98.1

Specific activities at 100% for : Free enzyme = 16.3 U/mg protein
Immobilized enzyme = 15.4 U/mg protein
Modified enzyme = 12.8 U/mg protein

The results in figure (5), recorded that both immobilized and modified enzymes were optimally at a higher temperature of 80°C than the free enzyme (70°C). Tanaksal *et al.* (2001) reported that the optimum temperature for *Conidiobolus macrosporus* alkaline protease was increased from 40 to 50°C after immobilization. The optimum temperature for trypsin activity was increased by 10°C when adding the β-cyclodextrin containing polysaccharide Fernandez *et al.* (2004). This fact suggests that the conformational rigidity to the protease was increased after the attachment to the oligosaccharide moieties, then requiring more temperature to express its maximum catalytic activity. Arrhenius plots of temperature data of the three forms of enzyme were linear. The calculated of activation energy (E_A) by the relationship:

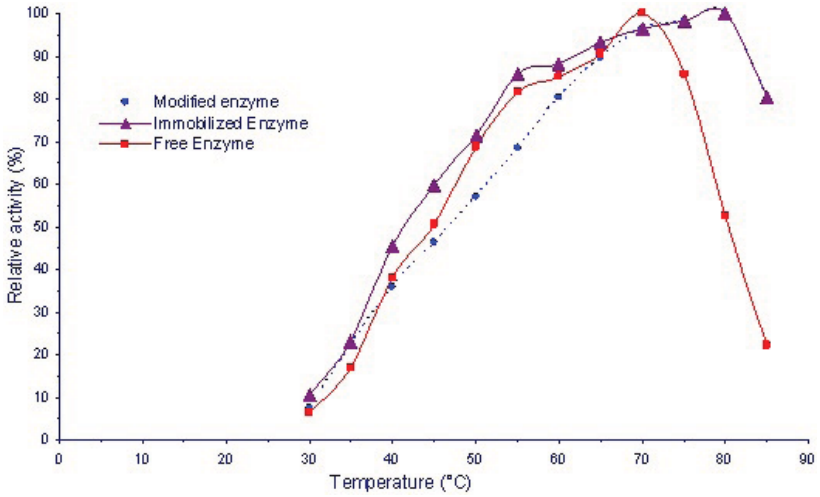

Fig. 5: Effect of temperature assay on the activity of B. licheniformis ATCC21415 alkaline protease.

- Slope = activation energy/ 2.303R where R is the gas constant (1.976 cal/mol)
- Activation energy of free, immobilized and, modified enzymes were 23.6, 18.9 and 19.9 Kcal/mol, A respectively. Abdel-Naby *et al.*, (1999) reported that (E) of modified enzyme was lower due to theenzyme stabilization by glycosylation.

Modified enzyme in comparison with the free enzyme (Figure 6) showed no change in the pH optimum (10.0), where the pH optimum for immobilized enzyme was 10.5. Gusek *et al.* (1990) found that immobilization shifted the pH activity profile to more alkaline pH values with an optimal activity at a higher pH (9.4) compared to the free enzyme (9.0).

The K_m and V_{max} values were determined from Linweaver-Burk plots of the (Figure 7 and 8). K_m of immobilized and modified enzymes were 5.0 and 5.3 mg/ml respectively which were higher than the free enzyme 4.8 mg/ml. Increasing the K_m value after immobilization about 10- fold was reported by Ohmiya *et al.* (1978).

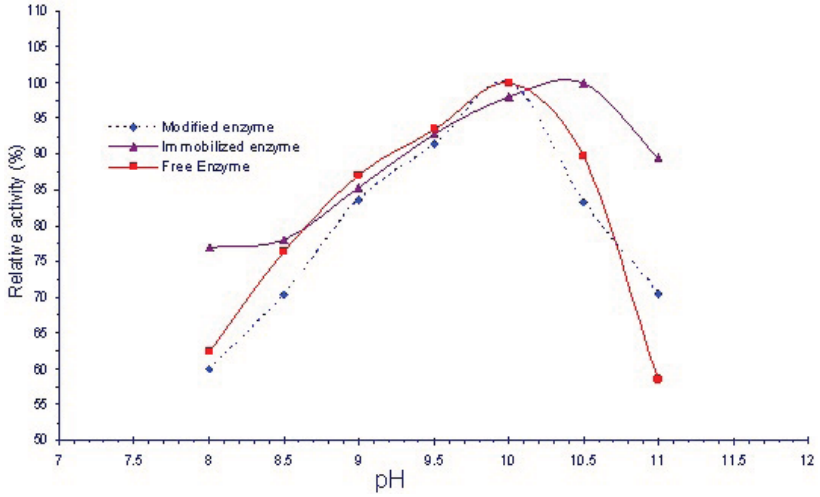

Fig. 6: Effect of pH on the activity of B. licheniformis ATCC21415 alkaline protease.

The increase of the K_m may be due to mass transfer resistance of the substance accessibility to the enzyme active site. The calculated maximal reaction rate (V_{max}) of the free enzyme was 22.7U/mg protein which higher than those of the immobilized and modified enzymes (20.0 and16.9U/mg protein), respectively. Decreasing the

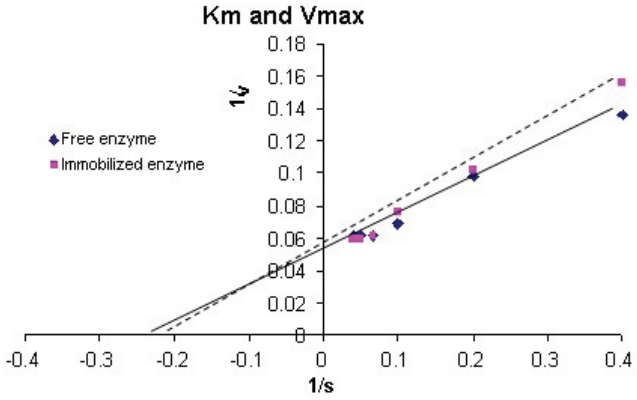

Fig. 7: Lineweaver-Burk plot for the free and immobilized enzyme.

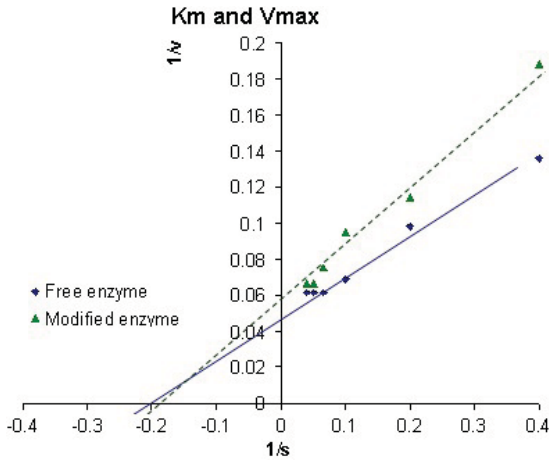

Fig. 8: Lineweaver-Burk plot for the free and immobilized enzyme.

V_{max} after immobilization and modification was similarly reported by Wehidy (2005) and Abdel-Naby *et al.* (1999). This decrease might be due to the fixation of the enzyme on the immobilization carrier which in turn and might be lead to decrease in the flexibility of the enzyme molecule which is commonly reflected by a decrease in the catalytic activity.

The enzyme activity was assayed after incubating the enzyme with various ions, inhibitors and detergents (10mM) at 37°C for 15min (Table 5). Most of the metal ions tested had a stimulator effect (Ca^{2+}, Co^{2+}, Mg^{2+}, $Mn^{2+}$and Na^{1+}) on the activity of free, immobilized and modified enzymes. On the other hand, Cu^{2+}ion

Table 5: Effect of some metal ions on the activity of *B. licheniformis ATCC21415* alkaline protease.

Metal ion (10mM)	Relative activity (%)		
	Free enzyme	Immobilized enzyme	Modified enzyme
$CaCl_2$	170.5	125.2	121.5
$CoCl_2$	149.4	130.1	125.9
$CuSO_4.5H_2O$	34.3	73.8	74.8
$MgSO_4.7H_2O$	167.4	113.2	108.3
$MnCl_2.4H_2O$	127.4	134.7	141.9
$NaCl_2$	178.1	110.3	102.5
EDTA	43.4	87.5	86.8
IAA	76.8	92.0	93.9
Pba	88.4	91.0	90.0
SDS	77.2	93.9	94.3
None	100.0	100.0	100.0

decreased the enzyme activity. This inhibitory effect was higher for the free enzyme (65.7%) than the immobilized and modified enzymes (26.2 and 25.2%), respectively. The free enzyme was strongly inhibited (relative activity 43.4%) by Ethylene-Diamine-Tetra- Acetic acid (EDTA) compared to the immobilized or modified enzyme (87.5 and 86.8%), respectively. The results suggested that the immobilization and modification protected the enzyme against the inhibitory effects of some metal ions and inhibitors. These results are in agreement with those reported for other enzymes Abdel-Naby *et al*. (1999). The latter authors reported that the glycosylation of enzyme formed stabile covalent bond that led to achievement of resistance against chemical.

The ability of crude protease to digest some natural proteins was tested (Figure 9) and showed that the enzyme can convert the insoluble forms of human clot and coagulated white egg to soluble form. The enzyme also was able to digest chicken skin after incubation for a long time with it. The results suggesting usefulness of this enzyme for different application such as extraction of collagen from skin for collagen replacement therapy, waste treatment and other related application.

Fig. 9: Effect of crude enzyme on some natural proteins.

Conclusion:

Loofa is a new carrier for *B. licheniformis* ATCC21415 alkaline protease immobilization by physical adsorption with the highest immobilization yield (70.5%). Chemical modification of protease was performed by covalent coupling with sodium periodate- activated amylopectin retained (78.3%). Immobilization and modification protect enzyme against temperature and inhibitors. The crude enzyme can digest some natural proteins and was able to extract collagen from chicken skin.

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