Kinetic, Catalytic and Thermodynamic Properties of Immobilized Milk Clotting Enzyme on Activated Chitosan Polymer and its Application in Cheese Making

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Abstract

Milk clotting enzyme (MCE) from *Bacillus circulans* 25 was immobilized by covalent binding, ionic binding and entrapment methods using various carriers. MCE covalently immobilized on activated chitosan polymer with the bifunctional agent glutaraldehyde (Ch-MCE) exhibited highest immobilization yield (74.6 %). Comparing to the native MCE, Ch-MCE exhibited higher optimum pH, higher optimum reaction temperature, lower activation energy, higher half-life time, lower deactivation rate constant and higher energy for denaturation. After immobilization, maximum reaction rate, Michaelis-Menten constant, specificity constant, turnover number, and catalytic efficiency of the enzyme were significantly changed. Calculated thermodynamic parameters for denaturation (enthalpy, entropy and Gibbs free energy) confirmed that the catalytic properties of MCE were significantly improved after immobilization. Reusability tests showed that after 7 catalytic cycles, the Ch-MCE retained about 71 % of its activity confirming its suitability for industrial applications.

Introduction

Using rennet enzyme in making cheese is the largest application of enzymes in food processing. Rennin acts in two stages for milk protein coagulation by specific hydrolysis of peptide bond (Phe$_{105}$-Met$_{106}$) of κ-casein (da Silva 2017). Recently, there are additional applications of proteases in dairy technology, to accelerate the ripening of cheese and to modify its functional properties (Afroz et al. 2015).

Microbial rennin is more acceptable in cheese production as an alternative to chymosin from newborn ruminants due to ethical problems and increased demand for cheese making (da Silva 2017).

Enzymes as bioactive agents have unique characteristics but the effectiveness is limited by their physico-chemical properties as stability (Ephrem et al. 2018). The use of soluble enzymes has some drawbacks that increase the consumption of enzymes as instability, easy inactivation, reduction of catalytic stability and difficulty of removal from the mixtures. To solve these problems, enzyme immobilization considered an effective technique which not only stabilizes enzymes under operating conditions but also allows easy recovery and reuse (Wehaidy et al. 2018).

Enzymes can be immobilized by adsorption, entrapment, covalent binding and ionic binding methods. However, immobilization by covalent binding is the most effective procedure in establishing enzymes and inhibiting their leakage due to the formation of strong covalent bond between carrier and enzyme (Eskandarloo and Abbaspourrad 2018). Covalent binding consists of two steps, first one, activation of functional groups found on carrier surface by a specific reagent, and the second, adding enzyme to form covalent bond with activated surface of carrier. In the coupling reaction, these activated groups will react with strong electron donating nucleophiles, such as the amino group (NH$_2$) and functional groups of certain amino acids on the surface of most enzymes (such as carboxylic group (COOH) of aspartic acid, amino groups (NH$_2$) of lysine, hydroxyl group (OH) of serine, and sulfhydryl group (SH) of cysteine).
Immobilization can be performed using different carriers whose properties play an important role in enzyme behavior. Desired properties of the insoluble carriers include low-cost, non-toxic, high surface area, reusable and good stability (mechanical, chemical and thermal). (Narwal et al. 2016). Since there is no universal carrier suitable for all enzymes and all applications, it is important to examine different carriers using different methods of enzyme immobilization. Various carriers have been used for immobilization e.g. polyacrylic (Esposito et al. 2016), alginate-pectate (Narwal et al. 2016), wool (Ahmed et al. 2018), basalt (Ahmed et al. 2019) and magnetic chitosan (Abdella et al. 2020).

Chitosan is one of natural polymer (polysaccharides) derived from chitin by deacetylation process and has excellent biocompatibility, no toxicity, cheapness, high mechanical strength, and a susceptibility to chemical modifications (Cahyaningrum and Sianita 2014; Salazar-Leyva et al. 2017). One common approach for enzyme immobilization on chitosan is through multipoint covalent binding between the functional groups present on the surface of an activated chitosan by cross-linking agents such as glutaldehyde and the surface functional groups of the enzyme protein.

Thermodynamics act as a key tool to understand the thermal deactivation process. Estimation of the thermodynamic parameters of the enzyme as enthalpy ($\Delta H^*$), entropy ($\Delta S^*$), and the Gibbs free energy ($\Delta G^*$) can provide useful information as enzyme behavior, activity and thermostability. The suitability of enzymes for industrial application is judged by their thermodynamic parameters (Zaboli et al. 2019).

The objectives of this study are to promote Bacillus circulans 25 MCE properties by covalent coupling to chitosan after activation and evaluate its catalytic, kinetics and thermodynamic parameters. Finally, it evaluates the reusability of Ch-MCE in cheese making.

**Materials And Method**

**Materials**

All the chemical reagents used were of analytical grade. Ceramic, wool and chicken bones were collected from the local market. Milk powder, skim milk powder spray dried (heat treated grade) was made in USA and obtained from the Ministry of Agriculture, Giza. Egypt.

**Methods**

**Enzyme production**

Milk clotting enzyme from Bacillus circulans 25 has been produced according to the previous work (Ahmed et al. 2018). The medium used for MCE production had the following composition (g/L): lactose 20, yeast extract 1, peptone 1, K$_2$HPO$_4$ 2 and MgSO$_4$.7H$_2$O 0.25. The pH was adjusted to 6.0 prior to sterilization. One mL of cell suspension of 24 h-old slant (OD600 ~ 0.3) was transferred to 50 mL sterile medium in 250-mL Erlenmeyer flask. The flasks were incubated at 35°C on a rotary shaker at 180 rpm for
24 h. The broth media after incubation was centrifuged at 6000 x g and 4°C for 15 min and the cell free filtrate was considered as source of crude enzyme.

**Milk clotting activity**

Milk clotting activity was estimated according to Narwal et al. (2016) method. Enzyme solution (2.5 mL) or certain weight of Ch-MCE was incubated with 10 mL skim milk (12 g dry skim milk/100 mL of 0.01 M CaCl₂) at 40°C. The end point is recorded when discrete particles were discernible by stop watch. One unit of the MCE activity (U) was equalized to 10 mL milk clotted within 10 min.

**Protein determination**

The protein content of the MCE preparation was estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The amount of immobilized protein was calculated by subtracting the amount of unbound protein from the amount of protein originally added for immobilization.

**Enzyme immobilization**

*B. circulans* 25 MCE was immobilized on various carriers by different methods of immobilization. The best carrier that exhibited highest immobilization yield (IY %) and highest immobilization efficiency (IE %) was chosen and used through this study. IY (%) and IE (%) were calculated according to Abdella et al. (2020) as following in Eq. (1, 2).

\[
\text{Immobilization yield} \, (\%) = \frac{I}{(A-B)} \times 100 \quad (1)
\]

\[
\text{Immobilization efficiency} \, (\%) = \left( \frac{I}{A} \right) \times 100 \quad (2)
\]

Where: I is the total activity of immobilized enzyme, A is the total activity offered for immobilization and B is the total activity of unbounded enzyme.

**Immobilization of MCE by covalent-binding**

The beads of chitosan were prepared by shaking 0.4 g chitosan in 5 mL of 0.01 M HCl containing glutaldehyde (GA 2.5 %) at 30°C for 2 h. The beads were precipitated using 0.1 N NaOH. The beads were collected by filtration, washed with distilled H₂O (to remove the excess GA). Then 5 mL enzyme solution (440U) was mixed with the wet beads by gently shaking. After 2 h at 30°C, the unbounded enzyme was removed by washing with distilled H₂O until no activity was detected. One gram of other carriers (chitin, wool, chicken bone, As-alumna, ceramic or PVC) was shaken in 25 mL Tris–HCl buffer (0.01 M, pH 6.0) containing 2.5 % GA at 30°C for 2 h. The carriers were filtered off, and washed with distilled H₂O to remove the excess GA. Then each treated carrier was incubated with Tris HCl buffer (5 mL, 220 U of MCE). After incubation at 30°C for 2 h, the unbounded enzyme was removed by washing with distilled H₂O (Abdel-Naby et al. 1998).

**Immobilization of MCE by ionic-binding**


The anion or cation exchanger (1 g) equilibrated with phosphate buffer (0.1 M, pH 6.0) or Tris-HCl (0.1 M, pH 8.0) and was incubated for 16 h at 4°C with certain volume of the enzyme solution (50 U) in the same buffer (Eskandarloo and Abbaspourrad 2018). The unbounded enzyme was removed by washing with the same buffer.

**Immobilization of MCE by alginate entrapment**

In this experiment, 10 mL of different concentrations of Na-alginate solution were mixed with equal volume of enzyme solution (200 U) to obtain final concentration range of 2–8 % (w/v). The whole mixture obtained by sodium alginate was extruded drop wise through a Pasteur pipette into a gently stirred 0.1 M CaCl$_2$ solution for 2h. The resulting beads with a diameter of ~ 1.0-1.5 mm were collected, washed with buffer and kept for 24 h at 4°C to remove the unbound enzyme (Dey et al. 2003).

**Characterization of free and chitosan immobilized MCE**

**Optimum pH and pH stability**

The effect of pH on the activity of free and Ch-MCE was investigated in 0.01 M buffer with different pH values (4.5–8.5). The relative activity was calculated according to Eq. (3).

Relative activity (%) = \( \frac{A_1}{A_2} \times 100 \) (3)

Where: A1 is the activity detected under the certain condition and A2 is the activity detected under the optimal condition.

The stability to pH was investigated by pre-incubating enzyme samples in 0.01M tris - HCl buffer with pH ranging from 5.0 to 9.0 at 25°C for 1h followed by adjusting the pH to the optimal of each enzyme form. The residual (retained) activity was assayed under the standard conditions and calculated to according to Eq. (4).

Residual activity (%) = \( \frac{A_f}{A_i} \times 100 \) (4)

Where: Af is the final activity detected and Ai is the initial activity detected.

**Optimum temperature and thermal stability**

The effect of temperature on the free and Ch-MCE was also determined. The enzyme samples in 0.01M tris - HCl buffer at pH 6.0 and 7.0 (for free and Ch-MCE), respectively were subjected to different temperatures (from 30°C to 100°C). The activation energy \( E_a \) was estimated from the slope of Arrhenius plot of log the residual enzyme activity (%) against reciprocal of absolute temperature in Kelvin (°K) according to Eq. (5).

\[ \text{Slope} = - \frac{E_a}{2.303 R} \] (5)

Where, \( E_a \) is the activation energy and R is the gas constant (1.976 Kcal/ mol).
Temperature coefficient value \( Q_{10} \), the rate of an enzymatic catalysis reaction changes for every 10°C rise in temperature, was calculated as reported by Wehaidy et al. (2018) as Eq. (6).

\[
Q_{10} = \text{antilog } E = (E \times 10^{RT^2}) \quad (6)
\]

Where \( E = E_a \) = activation energy

For thermal stability, free and Ch-MCE were heated at different temperatures (40–80°C) in the absence of substrate for different time intervals (15–120 min). Every 15 min a sample was removed and the residual activity was estimated under standard assay conditions. The enzyme activity without heating was taken as 100%. Deactivation rate constant \( k_d \) was determined according to Eq. (7) from the semi logarithmic plot of residual activity (%) versus time (min) (Singh et al. 2019).

\[
\text{Slope} = -k_d \quad (7)
\]

Half-life \( t_{1/2} \) value of inactivation is given according to Eq. (8).

\[
t_{1/2} = \ln 2 / k_d \quad (8)
\]

The energy for denaturation of enzyme \( E_{ad} \) was calculated from Arrhenius plot of \((\ln k_d)\) as a function of \((1/T)\) temperature in Kelvin \(^{\circ}K\) using the following in Eq. (9).

\[
\text{Slope} = - \frac{E_{ad}}{R} \quad (9)
\]

**Effect of substrate concentration**

Both the free and Ch-MCE activities were assayed with different substrate concentrations ranged from 1 to 12 % (w/v) at optimal assay conditions. Michaelis–Menten constant \( K_m \) and maximum velocity \( V_{max} \) were estimated from Lineweaver and Burke (1934). In addition, the turnover number \( k_{cat} \), catalytic efficiency \( k_{cat}/K_m \), specificity constants \( V_{max}/K_m \), free energy of substrate binding \( \Delta G_{E-S}^* \) and free energy of transition state binding \( \Delta G_{E-T}^* \) were estimated according to Abdel-Naby et al. (2015) and Wehaidy et al. (2018) as Eq. (10–15). :

\[
K_{cat} = \left( \frac{k_b T}{h} \right) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)} \quad (10)
\]

Where: \( k_b \) is Boltzmann's constant \((R/N) = 1.38 \times 10^{-23} \) J/ K, \( T \) is absolute temperature \((^\circ K)\), \( h \) is Planck's constant \( = 6.626 \times 10^{-34} \) Js, \( N \) is Avogadro's number \( = 6.02 \times 10^{23} \) / mol, \( R \) is Gas constant \( = 8.314 \) J/ K/ mol.

\[
\Delta H^* (\text{Enthalpy}) = E_a - RT \quad (11)
\]

\[
\Delta G^* (\text{Gibbs free energy of activation}) = -RT \ln (k_{cat} h / k_b x T) \quad (12)
\]

\[
\Delta S^* (\text{Entropy}) = (\Delta H^* - \Delta G^*) / T \quad (13)
\]
\[ \Delta G^*_{E-S} \text{ (Free energy of substrate binding)} = -RT \ln K_a, \text{ where } K_a = 1/k_m \quad (14) \]

\[ \Delta G^*_{E-T} \text{ (Free energy for transition state formation)} = -RT \ln \left( \frac{k_{cat}}{K_m} \right) \quad (15) \]

**Effect of metal ions**

The metal ions (ZnSO\(_4\), CoCl\(_2\), CaCl\(_2\), MnSO\(_4\), CuSO\(_4\), MgSO\(_4\), HgCl\(_2\), and NaAsO\(_2\)) were added individually (10 mM) to the reaction mixture. Both free and Ch-MCE activities were assayed under optimal assay conditions.

**Suitability of Ch-MCE in the making of cheese (reusability)**

A weight sample (4 g) of Ch-MCE (wet) was placed in a bag of muslin. The bag was immersed in skim milk solution (10 mL). The mixture was incubated at 85°C until forming the colt. At the end of the reaction, the bag containing the immobilized MCE was removed from the colt, washed with distilled water, and re-suspended in a freshly prepared substrate (10 mL) to start a new run.

**Results And Discussion**

*B. circulans* 25 MCE was immobilized on various carriers by different methods of immobilization to select the suitable carrier and method. The efficiency of enzyme immobilization was evaluated by different parameters including the residual catalytic activity (RA %), the specific activity (of the immobilized enzyme), and the immobilization efficiency (IE %). Moreover the immobilization yield (IY %) is the key parameter that it represents the general output of the immobilization process efficiency.

**Enzyme immobilization**

**Immobilization of MCE by covalent- binding**

Immobilization of MCE by covalent-binding was achieved by cross-linking between the enzyme and activated carriers throughout GA as a spacer group. The reaction happened between the NH\(_2\) groups found in the enzyme protein molecule and the free C = O group located on GA (the cross-linker) forming C = N- bond as reported by Abdella et al. (2020). The amount of MCE used for chitosan was higher than that used for other carriers due to its higher loading efficiency. The data presented in Table 1 indicated good IY and LE especially with chitosan (74.6 % and 315.0 U/g carrier). Our result is higher by 1.1-fold than that obtained by Esposito et al. (2016) on immobilized MCE. Covalent binding is the best method regarding to the strength of the interactions, reduce protein leakage (due to the formation of stable cross-linking between the enzyme and the carrier via spacer group). In addition, side chains of several amino acids can form covalent bonds with carriers (Nwagu et al. 2011). Moreover, covalent binding via spacer group may increase the local surface area and consequently reduced the protein crowding. Further, chitosan was preferred as a suitable carrier because it is cationic, biodegradable, inert, non-toxic and biocompatible compound (Pervez et al. 2017).
Table 1
Immobilization of *B. circulans* 25 MCE by covalent-binding

| Carrier          | Added enzyme (U/g) | Un bounded enzyme (U/g) | Immobilized enzyme (U/g) | Immobilization yield (IY %) |
|------------------|--------------------|-------------------------|--------------------------|-----------------------------|
|                  | (A)                | (B)                     | (I)                      |                             |
| Chitin           | 220.00             | 100.00                  | 79.20                    | 66.00                       |
| Wool             | 220.00             | 131.40                  | 49.75                    | 56.15                       |
| Chicken bones    | 220.00             | 133.30                  | 57.75                    | 66.61                       |
| Ceramic          | 220.00             | 122.40                  | 51.06                    | 52.31                       |
| As-alumina       | 220.00             | 118.32                  | 60.16                    | 59.17                       |
| PVC              | 220.00             | 131.92                  | 54.89                    | 62.32                       |
| Chitosan         | 440.00             | 17.60                   | 315.00                   | 74.57                       |

**Immobilization of MCE by ionic- binding**

A series of ion exchangers was used for the immobilization of MCE by ionic binding (Table 2). As shown by the results, Dowex 50W was the most suitable ion exchanger for MCE immobilization with highest loading efficiency (LE 19.0 U/g carrier) and highest IY (56.7 %). On the contrary, MCE immobilized on DEAE-Sephdex A-25 had no activity. This inhibition of the binding enzyme with DEAE-Sephdex A-25 may be due to that the enzyme's active sites being involved in the fixation process. Similarly, Abdel-Naby et al. (1998) pointed to the low bound enzyme for protease immobilization by ionic binding.

Table 2
Immobilization of *B. circulans* 25 MCE by ionic- binding

| Carrier           | Added enzyme (U/g) | Un bounded enzyme (U/g) | Immobilized enzyme (U/g) | Immobilization yield (IY %) |
|-------------------|--------------------|-------------------------|--------------------------|-----------------------------|
|                   | (A)                | (B)                     | (I)                      |                             |
| Dowex 50 W        | 50.00              | 16.40                   | 19.00                    | 56.65                       |
| Dowex 1x4         | 50.00              | 17.10                   | 8.50                     | 25.84                       |
| Ambelite IR- 120  | 50.00              | 23.80                   | 10.00                    | 38.17                       |
| DEAE–Cellulose DE-52 | 50.00         | 39.80                   | 3.30                     | 32.35                       |
| DEAE –Sephadex A-25 | 50.00           | 26.30                   | -                        | -                           |

**Immobilization by alginate entrapment**
MCE was immobilized by entrapment in Ca-alginate with different concentrations (Table 3). The results showed that the LE was gradually decreased from 37.2 U/10 mL gel to 18 U/10 mL gel with the gel concentration increase from 2 to 8 %. This probably due to the decrease of the gel porosity with the increase of Na-alginate concentration, and consequently the diffusion limitation was developed. Similar observation was previously reported for entrapped proteases (Abdel-Naby et al. 1998; Lamas et al. 2001). The pore size of the gel, reflected in the viscosity of the carrier due to the size of the molecule and/or its concentration, can affect the diffusion of substrates or products and limit the reaction rates of the entrapped enzyme.

| Na–alginate concentration (%) | Added enzyme (U/10ml gel) (A) | Unbounded enzyme (U/10ml gel) (B) | Immobilized enzyme (U/10ml gel) (I) | Immobilization Yield (IY %) |
|-----------------------------|-------------------------------|----------------------------------|------------------------------------|---------------------------|
| 2                           | 200.00                        | 9.00                             | 37.20                              | 19.48                     |
| 4                           | 200.00                        | 11.60                            | 24.30                              | 12.89                     |
| 6                           | 200.00                        | 13.90                            | 21.10                              | 11.34                     |
| 8                           | 200.00                        | 15.30                            | 18.00                              | 9.74                      |

**Characterization of free and Ch-MCE**

Among all preparations, chitosan-immobilized MCE (Ch-MCE) had the highest IY and the highest LE, consequently, in the following experiments Ch-MCE was used.

**Specific activity**

Ch-MCE retained 77 % of the initial specific activity shown by the free enzyme. This retained activity is higher than that mentioned for the MCE immobilized on silica gel (El-Bendary et al. 2009). The activity of the enzyme may decrease as other proteins due to the corresponding changes caused by the immobilization (Siar et al. 2017). Also, the decrease in the specific activity may be due to the diffusional limitation of the substrate and product flow. In fact, immobilization carriers present problems in the mass transfer rate of the substrate and products not found with free enzymes. Although GA develops very strong links between enzymes and carriers it denatures the structure of some enzymes during the binding process, causing undesirable loss of activity (Salazar-Leyva et al. 2017).

**Effect of pH on the free and Ch-MCE activity**

As illustrated in Fig. 1 the free MCE was optimally active at pH 6.0, however, the Ch-MCE was optimally active at pH 7.0. At higher pH values up to 8.0, the drop in activity was more pronounced with the free than that of the Ch-MCE. These effects may be due to the changes of the ionic microenvironment of the enzyme active site and/or distribution of the surface charges of the carrier after immobilization (Talbert
and Goddard 2012; Pervez et al. 2017). These are due to the use of cationic carrier like chitosan which interacts with anionic groups on the enzyme surface. Pervez et al. (2017) reported that the pH in the immediate vicinity of enzyme molecule may change, depending upon the surface and residual charge on the solid support (chitosan is positively charged due to amino groups).

**pH stability of free and Ch-MCE**

The results in Fig. 2 showed that the pH stability of Ch-MCE was shifted to higher pH values compared to the free MCE. These results reveal the same trend as in the shift of the optimum pH. The results showed that covalent binding maintained the MCE catalytic activity especially in the case of alkalinity. Pervez et al. (2017) reported that immobilization decreases the inhibition of enzymes either by stabilizing the enzyme structure or by eliminating the inhibitor. Moreover, multipoint covalent attachment between the enzyme molecule and an activated carrier makes the structure of the enzyme more rigid, consequently decreasing conformational changes which can be induced by extreme pH (Salazar-Leyva et al. 2017). Narwal et al. (2016) suggested that titratable acidity of milk should be between 0.19 to 0.25 % lactic acid equivalents (which correspond to pH from 5.0 to 6.5) at the time of adding an enzyme. Therefore, for industrial applications, the MCE should be stable between this pH ranges.

**Effect of temperature on the free and Ch-MCE activity**

The optimal reaction temperature of the MCE (free and Ch-MCE) was investigated at their optimal pH. As seen in Fig. 3 and Table 4, the free enzyme was optimally active at 75°C however, the Ch-MCE was optimally active at 85°C. Increasing of the optimum temperature for Ch-MCE by 10 degrees is probably a consequence of enhanced thermal stability by immobilization. Covalent binding of enzymes onto chitosan enhanced the optimum temperature and stability of the biocatalysts for thermal inhibition (Salazar-Leyva et al. 2017). The calculated $E_a$ value of Ch-MCE was lower by 1.4-fold than that of the free MCE (Fig. 4 and Table 4) confirming that immobilization improved the catalytic efficiency of the enzyme by lowering the energy required to make the activated complex of enzyme and substrate (Wehaidy et al. 2018). The calculated $E_a$ was lower by 1.7-fold than that obtained for stabilized *Aspergillus fumigatus* protease (Hernandez-Marinez et al. 2011). Reduced $E_a$ of the immobilized enzyme compared to free enzyme may be due to the mass transfer limitations (Thakrar and Singh 2019).
Table 4  
Thermal properties of free and Ch-MCE

| Property                                      | Milk-clotting enzyme |   |   |
|-----------------------------------------------|----------------------|---|---|
|                                               | Free                | Immobilized |   |
| Optimum reaction temperature (°C)             | 75                   | 85 |   |
| Activation energy $E_a$ (KJ/mol)              | 36.76               | 34.31 |   |
| Deactivation rate constant $k_d$/min, at      |   |   |
| 60°C                                          | 8.35 x $10^{-3}$    | Stable for 120 min |   |
| 65°C                                          | 13.5 x $10^{-3}$    | 1.97 x $10^{-3}$ |   |
| 70°C                                          | 21.167 x $10^{-3}$  | 4.45 x $10^{-3}$ |   |
| 75°C                                          | Unstable            | 10.09 x $10^{-3}$ |   |
| Half life time $t_{1/2}$ (min), at            |   |   |
| 60°C                                          | 36.04                | Stable for 120 min |   |
| 65°C                                          | 22.29                | 152.79 |   |
| 70°C                                          | 14.22                | 66.88 |   |
| 75°C                                          | Unstable             | 29.83 |   |
| Energy for denaturation $E_{ad}$ (KJ/mol)     | 217.34               | 378.73 |   |

**Thermal stability of free and Ch-MCE**

Although thermostable enzymes are more suitable for industrial applications than mesophilic enzymes, stability of MCEs for long times at a mild temperatures is very important for their suitability in making cheese. The results in Fig. 5 showed that the immobilization improved the stability of MCE to the thermal inhibition. Thus, after heating for 120 min, the Ch-MCE was stable up to 60°C with 100 % residual activity whereas, the free MCE lost about 90.4 % of its initial activity. In addition, after heat treatment at 70°C for 90 min, the free MCE was MCE Free was completely inhibited however, the Ch-MCE retained 40 % of its initial activity. The increase in enzyme stability after immobilization was possibly related to the higher rigidity of the immobilized form (Yang et al. 2017). In addition, the stability to heat inhibition enhancement after immobilization could be caused by the carrier that protects the enzyme from denaturation by absorbing a great amount of heat (Figueira et al. 2011).

The calculated values of deactivation rate constant $(k_d)$ in Table 4 indicated that the stability of the Ch-MCE to the thermal inhibition was superior to that of the free MCE (the lower $k_d$, the more thermo stable...
enzyme). For example, $k_d$ at 70°C for the free enzyme was $13.6 \times 10^{-3}$, which was higher by 3-fold than that of the Ch-MCE ($4.4 \times 10^{-3}$). These results confirm the effectiveness of the MCE immobilization on chitosan for increasing the thermal stability. In addition, half-life ($t_{1/2}$) of the Ch-MCE at 65, and 75°C were higher by 6 and 5-fold, respectively than that of the free MCE. The binding between the enzyme and the carrier reduces conformational flexibility and thermal vibration, thus protecting the immobilized protein from denaturing and unfolding by increasing the temperature (Figueira et al. 2011).

The energy for thermal denaturation ($E_{ad}$) for Ch-MCE was greater by 161.39 kJ/mol than that obtained for free MCE (Fig. 6 and Table 4). This result indicated that immobilized form needed more energy for deactivation compared to the free form. The energy of thermal inactivation is an important factor to judge its thermal stability (Thakrar and Singh 2019).

**Effect of substrate concentration**

As seen in Lineweaver-Burk plots (Fig. 7) Ch-MCE provided $k_m$ 1.3-fold higher and $V_{max}$ 1.3-fold lower than the free MCE. In addition, the $V_{max}/K_m$ of Ch-MCE was decreased by 1.6-fold compared to the free MCE (Table 5). This change in enzyme affinity towards its substrate after immobilization probably related to the low substrate accessibility to the enzyme active sites due to the changes in active sites of enzyme introduced by bonding with GA activated carrier (Pervez et al. 2017). Low $V_{max}/K_m$ ratio specifies the smaller specificity of immobilized form for casein than the free MCE (Singh et al. 2019). Similar observation was reported by (Pal and Khanum 2011; Cahyaningrum and Sianita 2014) concerning the free and immobilized xylanase and pepsin. In addition, decreased $K_m$ after immobilization seems due to the resistance to mass transfer and the reduction of the immobilized enzyme flexibility to its substrate (Thakrar and Singh 2019). The kinetic and thermodynamic parameters of substrate hydrolysis by the free and the Ch-MCE were presented in Table 5. The turnover number ($K_{cat}$) of Ch-MCE was higher by 1.5-fold compared to the free MCE. $K_{cat}$ represents the maximum number of reactions catalyzed per minute. The catalytic efficiency ($K_{cat}/K_m$) of free and Ch-MCE were 0.154 and 0.174 /S mg/ mL, respectively, indicating that the immobilization technique enhanced the catalytic efficiency ~ 13 %. On contrary, Mafra et al. (2019) found that the immobilized enzyme catalytic efficiency was 34 % lower than the free form.
Table 5
kinetic and thermodynamic parameters for casein hydrolysis by free and Ch- MCE.

| Parameter                                                   | Milk-clotting enzyme |
|-------------------------------------------------------------|----------------------|
|                                                             | Free             | Immobilized    |
| $E_a$ (KJ/ mol)                                             | 36.76            | 34.31          |
| $V_{max}$ (U/ mg protein)                                  | 10.0             | 8.0            |
| $K_m$ (mg skim milk/ ml)                                   | 28.01            | 35.71          |
| $K_{cat}$ ($S^{-1}$)                                       | 4.3              | 6.23           |
| $K_{cat}/ K_m$ (/S mg/ ml)                                 | 0.1535           | 0.1744         |
| $K_a$ (1/Km)                                                | 0.036            | 0.028          |
| $\Delta H^*$ (kJ/ mol)                                     | 33.86            | 31.33          |
| $\Delta G^*$ (kJ/ mol)                                     | 96.68            | 102.17         |
| $\Delta S^*$ (J/ mol /K)                                   | -183.67          | -197.87        |
| $\Delta G^*_{E-T}$ ((kJ/ mol)                              | 5.34             | 5.12           |
| $\Delta G^*_{E-S}$ ((kJ/ mol)                              | 9.48             | 10.5           |
| $V_{max} / K_m$                                            | 0.357            | 0.224          |
| $Q_{10}$                                                    | 1.0              | 1.0            |

The thermodynamic parameters were calculated as following according to Wehaidy et al. (2018):

$$K_{cat} = (k_b T / h) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)}$$

Where: $k_b$ is Boltzmann's constant $(R/N) = 1.38 \times 10^{-23}$ J/ K, $T$ is Absolute temperature (°K),

$h$ is Planck’s constant $= 6.626 \times 10^{-34}$ Js, $N$ is Avogadro’s number $= 6.02 \times 10^{23}$ / mol,

$R$ is Gas constant $= 8.314$ J/ K/ mol.

$\Delta H^*$ (Enthalpy) = $E_a - RT$

$\Delta G^*$ (Gibbs free energy of activation) = -$RT \ln (k_{cat} h / k_b x T)$

$\Delta S^*$ (Entropy) = $(\Delta H^* - \Delta G^*) / T$

$\Delta G^*_{E-S}$ (Free energy of substrate binding) = -$RT \ln K_a$, where $K_a = 1/k_m$
The recorded data in Table 5 showed a decrease in the Ch-MCE enthalpy of activation (ΔH*) compared to the free MCE. In addition, the entropy (ΔS*) of activation for Ch-MCE was lower by 14.2 kJ/mol than that of the free MCE. The lower ΔH* and the negative ΔS* values for Ch-MCE pointed to the stability and the effective transitional state of the complex of enzyme-substrate (Wehaidy et al. 2018). Gibbs free energy (ΔG*) of a substance results from the stabilizing forces present in protein’s structure, such as Van der Waals interactions and hydrogen bonds. The higher ΔG* is associated with more tolerance toward heat inactivation (Zaboli et al. 2019). As shown in Table 5, ΔG* for the Ch-MCE was higher by 5.49 kJ/mol than the free MCE indicating that a change of the Ch-MCE-substrate complex into products is less spontaneous in comparison with free MCE. The catalytic reaction can be evaluated by estimating the Gibbs free energy (ΔG*) for enzyme-substrate complex conversion into products (Riaz et al. 2007).

The free energy of transition state formation (ΔG*E−T) for Ch-MCE (5.12 kJ/mol) was lower compared to the free MCE (5.34 kJ/mol). This result indicated that this reaction is more spontaneous for Ch-MCE than for free MCE (Ferreira et al. 2018). In addition the Gibbs free energy of substrate binding (ΔG*E−S) confirmed that the Ch-MCE requires higher amount of free energy (10.5 kJ/mol) to form this transition state compared to the free MCE (9.5 kJ/mol). The same behavior among soluble and conjugated MCE from B. subtilis KU710517 was reported by Wehaidy et al. (2018).

**Effect of metal ions**

The effects of various metal ions on the Ch-MCE activity compared to the free MCE were illustrated in Fig. 8. Both the free and the Ch-MCE were activated by Mg²⁺, Ca²⁺ and Mn²⁺ whereas As⁺², Hg⁺² and Co⁺² inhibited them. Cu⁺² decrease enzymatic activity of the free MCE by 8.7 % although no lose in activity of Ch-MCE. In general, it was observed that the inhibitory effect of these investigated metal ions was less pronounced with the Ch-MCE compared to free MCE (Pandey et al. 2017). This probably related to the protection of the immobilized enzyme by the carrier against the inhibitory effects of some chemicals. Siar et al. (2017) suggested that if multi-point covalent bond is achieved, the structure of enzyme becomes more rigid and consequently increases enzyme stability under any distorting reagent.

**Suitability of Ch-MCE in making cheese (reusability)**

The greatest immobilized enzymes advantage their repeated use which is of practical utility for commercial purposes as it can be easily separated from soluble reactants and products. The Ch-MCE retained about 100 and 90.4 % of the initial activity after being used for 5 and 7 consecutive cycles,
respectively. Multipoint covalent binding of enzymes on activated supports promotes a rigidification of its structure and reusability (Ahmed et al. 2019). Upon repeated use, gradual decrease in activity was observed (Fig. 9) which probably due to the enzyme denaturation and loss of the enzyme from the carrier physically. Conversely, Salazar-Leyva et al. (2017) reported that immobilized proteases retained 40 % of its initial activity after the second cycle.

Conclusion

*B. circulans* 25 milk clotting enzyme was successfully immobilized on activated chitosan (Ch-MCE) with immobilization yield 75% and immobilization efficiency 72%. Based on the results, thermal properties of the Ch-MCE were enhanced. Compared to the free enzyme, Ch-MCE exhibited higher optimum temperature, lower *kd* (deactivation rate constant) and higher *t*₁/₂ (half-life time). In addition, immobilization improved the quality of MCE by decreasing the activation energy (*Ea*) by 0.9-fold compared to the free enzyme. Moreover, the *E*ₐ₅ (energy for denaturation) of the Ch-MCE was 1.8-fold higher than that of the free enzyme meaning that the immobilization process increases the heat resistance of enzyme (more energy is required to enzyme denaturation). The calculated thermodynamic parameters as enthalpy (*ΔH*), Gibbs free energy (*ΔG*) and entropy (*ΔS*) demonstrated that covalent binding between enzyme and activated chitosan increased its thermal stability. Also, Ch-MCE showed higher pH stability at different pH values compared with the free enzyme. Ch-MCE is suitable for application in cheese making and can be used successfully for 8 consecutive cycles with 80 % residual activity. It can be concluded that this work helps to overcome the limitations of the reduction in MCE catalytic activity that associated with changes in temperature, pH and inhibitors making it useful in industrial applications and biotechnological process.

Declarations

Authors’ contributions

Abdel-Fattah; participated in studies. Abdel-Naby; supervised the complete study. Ahmed; performed the research experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data obtained or analyzed during this study are included in this article and available from the corresponding author.

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**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

The publication of the paper has been agreed by the authors.

**Competing interests**

The authors declare that they have no potential interest.

**References**

1. Abdella MAA, El-Sherbin GM, El-Shamy AR, Atalla SMM, Ahmed SA (2020) Statistical optimization of chemical modification of chitosan-magnetic nanoparticles beads to promote *Bacillus subtilis* MK1 α-amylase immobilization and its application. Bull of the NRC 44: 1–13. doi: 10.1186/s42269-020-00301-3

2. Abdel-Naby MA, Ismail AMS, Ahmed SA, Abdel-Fattah AF (1998) Production and immobilization of alkaline protease from *Bacillus mycoides*. Biores Technol 64: 205–210. https://doi: 10.1016/S0960-8524(97)00160-0

3. Abdel-Naby MA, Abdel-Fattah AF, Reyad RM (2015) Catalytic and thermodynamic properties of immobilized *Bacillus amyloliquefaciens* cyclodextrin glucosyltransferase on different carriers. J of Molec Cataly B: Enzy 116:140–147. doi 10.1016/j.molcatb.2015.03.011.

4. Afroz QM, Khan KA, Ahmed P, Uprit S (2015) Enzymes used in dairy industries. Inter J of Appl Rese 1(10):523–527

5. Ahmed SA, Abdel-Naby MA, Abdel-Fattah AF (2018) Applicability of wool covalent bonded *Bacillus circulans* 25 cells for milk-clotting enzyme production by batch, repeated batch and continuous process. Brazi J of Chem Engin 35:847–856. https://doi:10.1590/0104-6632.20180353s20170175

6. Ahmed SA, Abdel Wahab WA, Abdel-Hameed SAM (2019) Comparative study in kinetics and thermodynamic characteristics of immobilized caseinase on novel support from basalt by physical adsorption and covalent binding. Bioc Agric Biotec 18: 101028. https://doi.org/10.1016/j.bcab.2019.101028

7. Cahyaningrum SE, Sianita MM (2014) Immobilization of pepsin onto chitosan silica nanobeads with glutaraldehyde as crosslink agent. Bullet of Chem React Engin Cataly 9:263–269. https://doi:10.9767/bcroc.9.3.7060.263-269

8. da Silva RR (2017) Bacterial and fungal proteolytic enzymes: Production, catalysis and potential applications. Appl Bioch Biotech 183:1–19. https://doi:10.1007/s12010-017-2427-2
9. Dey G, Singh B, Banerjee R (2003) Immobilization of α-amylase produced by Bacillus circulans GRS313. Brazil Arch of Biol Technol 46:167–176

10. El-Bendary MA, Moharam ME, Ali TH (2009) Efficient immobilization of milk clotting enzyme produced by Bacillus sphaericus. Poli J of Food Nutr Sci 59:67–72

11. Ephrem E, Najjar A, Charcosset C, Greige-Gerges H (2018) Encapsulation of natural active compounds, enzymes, and probiotics for fruit juice fortification, preservation, and processing: An overview. J of Funct Food 48:65–84. https://doi:10.1016/j.jff.2018.06.021

12. Eskandarloo H, Abbaspourrad A Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food Chemis 251:115–124. https://doi:10.1016/j.foodchem.2018.01.068

13. Esposito M, Pierro PD, Dejonghe W, Mariniello L, Porta R (2016) Ezymatic milk clotting activity in artichoke (Cynara scolymus) leaves and alpine thistle (Carduus defloratus) flowers. Immobilization of alpine thistle aspartic protease. Food Chem 204:115–121. https://doi:10.1016/j.foodchem.2016.02.060

14. Ferreira MM, Santiago FLB, da Silva NAG, Luiz JHH, Fernández-Lafuente R, Mendes AA, Hirata DB (2018) Different strategies to immobilize lipase from Geotrichum candidum: Kinetic and thermodynamic studies. Proc Bioch 67:55–63. https://doi:10.1016/j.procbio.2018.01.028

15. Figueira Jde-A, Dias FFG, Sato HH, Fernandes P (2011) Screening of supports for the immobilization of β-glucosidase. Enzy Resea Article ID 642460. https://doi:10.4061/2011/642460

16. Hernandez-Mariinez R, Gutiérrez-Sanchez G, Bergmann CW, Loera-Corrall O, Rojo-Dominguez A, Huerta-Ochoa S, Regalado-Gonzalez C, Prado-Barragan LA (2011) Purification and characterization of a thermodynamic stable serine protease from Aspergillus fumigatus. Proc Bioch 46:2001–2006. https://doi:10.1016/j.procbio.2011.07.013

17. Lamas EM, Berros RM, Balcão VM, Malcata FX (2001) Hydrolysis of whey protein by protease extracted from Cynara cardunculus and immobilized into highly activated supports. Enz Microb Technol 28:642–652. https://doi:10.1016/s0141-0229(01)00308-8

18. Lineweaver H, Burke D (1934) Determination of enzyme dissociation constants. J Am Chem Soc 56:658–666. https://doi:10.1021/ja01318a036

19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

20. Mafra ACO, Ulrich LG, Kornecki JF, Fernandez-Lafuente R, Tardioli PW, Ribeiro MPdeA (2019) Combi-CLEAs of glucose oxidase and catalase for conversion of glucose to gluconic acid eliminating the hydrogen peroxide to maintain enzyme activity in a bubble column reactor. Cataly 9:1–16. https://doi:10.3390/catal9080657

21. Narwal RK, Bhushan B, Pal A, Malhotra S, Kumar S, Saharan V (2016) Inactivation thermodynamics and iso-kinetic profiling for evaluating operational suitability of milk clotting enzyme immobilized in composite polymer matrix. Inter J of Biol Macromol 91:317–328. https://doi:10.1016/j.ijbiomac.2016.05.025
22. Nwagu TN, Okolo BN, Aoyagi H (2011) Immobilization of raw starch digesting amylase on silica gel: A comparative study. Afric J of Biotech 10:15989–15997. https://doi:10.5897/AJB10.2346

23. Pal A, Khanum F (2011) Covalent immobilization of xylanase on glutaraldehyde activated alginate beads using response surface methodology: Characterization of immobilized enzyme. Proc Biochem 46:1315–1322. https://doi:10.1016/j.procbio.2011.02.024

24. Pandey G, Munguambe DM, Tharmavaram M, Rawtani D, Agrawal YK (2017) Halloysite nanotubes-An efficient nano-support for the immobilization of α-amylase. Appl Clay Sci 136:184–191. https://doi:10.1016/j.clay.2016.11.034

25. Pervez S, Aman A, Qader SAUl (2016) Role of two polysaccharide matrices on activity, stability and recycling efficiency of immobilized fungal amylglucosidase of GH15 family. Inter J of Biol Macromol 96:70–77. https://doi:10.1016/j.ijbiomac.2016.12.023

26. Riaz M, Perveen RR, Javed MR, Nadeem H, Rashid MH (2007) Kinetic and thermodynamic properties of novel glucoamylase from Humicola sp. Enz Microb Technol 41:558–564. https://doi:10.1016/j.enzmictec.2007.05.010

27. Salazar-Leyva JA, Lizardi-Mendoza J, Ramirez-Suarez JC, Lugo-Sanchez ME, Valenzuela-Soto EM, Ezquerra-Brauer JM, Castillo-Ya ~ Nez FJ, Pacheco-Aguilar R (2017) Catalytic and operational stability of acidic protease from Monterey sardine (Sardinops sagax caerulea) immobilized on a partially deacetylated chitin support. J of Food Biochem 41:e12287. https://doi:10.1111/jfbc.12287

28. Siar E, Zaak H, Komecki JF, Zidouneb MN, Barbosa O, Fernandez-Lafuente R (2017) Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins. Proc Biochem 58:98–104. https://doi:10.1016/j.procbio.2017.04.009

29. Singh RS, Chauhan K, Kennedy JF (2018) Fructose production from inulin using fungal inulinase immobilized on 3-aminopropyl-triethoxysilane functionalized multiwalled carbon nanotubes. Inter J of Biol Macromol 125:41–52. https://doi:10.1016/j.ijbiomac.2018.11.281

30. Talbert JN, Goddard JM (2012) Enzymes on material surfaces. Coll Sur B Biointer 93:8–19. https://doi:10.1016/j.colsurfb.2012.01.003

31. Thakrar FJ, Singh SP (2019) Catalytic, thermodynamic and structural properties of an immobilized and highly thermostable alkaline protease from a haloalkaliphilic actinobacteria, Nocardiosis alba TATA-5. Biorees Technol 278:150–158. https://doi:10.1016/j.biortech.2019.01.058

32. Wehaidy HR, Abdel-Naby MA, Shousha WG, Elmallah MIY, Shawky MM (2018) Improving the catalytic, kinetic and thermodynamic properties of Bacillus subtilis KU710517 milk clotting enzyme via conjugation with polyethylene glycol. Inter J of Biol Macromol 111:296–301. https://doi:10.1016/j.ijbiomac.2017.12.125

33. Yang A, Long C, Xia J, Tong P, Cheng Y, Wang Y, Chen H (2017) Enzymatic characterization of the immobilized alcalase to hydrolyse egg white protein for potential allergenicity reduction. J Sci Food Agric 97:199–206. https://doi:10.1002/jsfa.7712
34. Zaboli M, Raissi H, Zaboli M, Farzad F, Torkzadeh-Mahani M (2019) Stabilization of D-lactate dehydrogenase diagnostic enzyme via immobilization on pristine and carboxyl-functionalized carbon nanotubes, a combined experimental and molecular dynamics simulation study. Arch of Biochem Biophy 661:178–186. doi 10.1016/j.abb.2018.11.019.

**Figures**

**Figure 1**

Effect of pH on the activity of free MCE (hollow) and Ch-MCE (solid)
Figure 2

pH stability of free MCE (hollow) and Ch-MCE (solid)
Figure 3

Effect of temperature on the activity of free MCE (hollow) and Ch-MCE (solid)
Figure 4

Arrhenius plot for temperature dependence of the activity of free MCE (hollow) and Ch-MCE (solid)
Figure 5
First-order plots of the effect of thermal inactivation of free MCE (hollow) and Ch-MCE (solid)

Figure 6
Arrhenius plot for activation energy of denaturation for free MCE (hollow) and Ch-MCE (solid)
Figure 7

Lineweaver-Burk Plot for free MCE (hollow) and Ch-MCE (solid)
Figure 8

Effect of metal ions on the activity of free MCE (hollow) and Ch-MCE (solid)
Figure 9

Effect of metal ions on the activity of free MCE (hollow) and Ch-MCE (solid)

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