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 (Phe₁₀₅-Met₁₀₆) 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 (NH₂) and functional groups of certain amino acids on the surface of most enzymes (such as carboxylic group (COOH) of aspartic acid, amino groups (NH₂) 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 (ΔH^*), entropy (ΔS^*), and the Gibbs free energy (ΔG^*) 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_2HPO_4 2 and $MgSO_4 \cdot 7H_2O$ 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 CaCl_2) 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).

$$\text{Immobilization yield (\%)} = I / (A - B) \times 100 \quad (1)$$

$$\text{Immobilization efficiency (\%)} = (I/A) \times 100 \quad (2)$$

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 H_2O (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 H_2O 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 H_2O 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 H_2O (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 unbound 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).

$$\text{Relative activity (\%)} = (A1/A2) \times 100 \quad (3)$$

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 according to Eq. (4).

$$\text{Residual activity (\%)} = (A_f / A_i) \times 100 \quad (4)$$

Where: A_f is the final activity detected and A_i 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).

$$\text{Slope} = - E_a / 2.303 R \quad (5)$$

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 (°K) using the following in Eq. (9).

$$\text{Slope} = -E_{ad} / R \quad (9)$$

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 (ΔG^*_{E-S}) and free energy of transition state binding (ΔG^*_{E-T}) were estimated according to Abdel-Naby et al. (2015) and Wehaidy et al. (2018) as Eq. (10–15). :

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

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

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

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

$$\Delta S^* (\text{Entropy}) = (\Delta H^* - \Delta G^*) / T \quad (13)$$

ΔG^*_{E-S} (Free energy of substrate binding) = $-RT \ln K_a$, where $K_a = 1/k_m$ (14)

ΔG^*_{E-T} (Free energy for transition state formation) = $-RT \ln (k_{cat}/K_m)$ (15)

Effect of metal ions

The metal ions (ZnSO₄, CoCl₂, CaCl₂, MnSO₄, CuSO₄, MgSO₄, HgCl₂ and NaAsO₂) 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 NH₂ 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) (A)	Un bounded enzyme (U/g) (B)	Immobilized enzyme (U/g) (I)	Immobilization yield (IY %)
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-Sephadex A-25 had no activity. This inhibition of the binding enzyme with DEAE-Sephadex 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) (A)	Un bounded enzyme (U/g) (B)	Immobilized enzyme (U/g) (I)	Immobilization yield (IY %)
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.

Table 3
Immobilization of *B. circulans* 25 MCE by entrapment in Ca-alginate

Na – alginate concentration (%)	Added enzyme (U/10ml gel) (A)	Un bounded 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-Mariinez 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	8.35×10^{-3}	Stable for 120 min
65°C	13.5×10^{-3}	1.97×10^{-3}
70°C	21.167×10^{-3}	4.45×10^{-3}
75°C	Unstable	10.09×10^{-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 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 13.6×10^{-3} , which was higher by 3-fold than that of the Ch-MCE (4.4×10^{-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/ K_m)	0.036	0.028
ΔH^* (kJ/ mol)	33.86	31.33
ΔG^* (kJ/ mol)	96.68	102.17
ΔS^* (J/ mol /K)	-183.67	-197.87
ΔG^*_{E-T} ((kJ/ mol)	5.34	5.12
ΔG^*_{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) = 1.38×10^{-23} J/ K, T is Absolute temperature (°K),		
h is Planck's constant = 6.626×10^{-34} Js, N is Avogadro's number = 6.02×10^{23} / mol,		
R is Gas constant = 8.314 J/ K/ mol.		
ΔH^* (Enthalpy) = $E_a - RT$		
ΔG^* (Gibbs free energy of activation) = $-RT \ln (k_{cat} h / k_b \times T)$		
ΔS^* (Entropy) = $(\Delta H^* - \Delta G^*) / T$		
ΔG^*_{E-S} (Free energy of substrate binding) = $-RT \ln K_a$, where $K_a = 1/k_m$		

Parameter	Milk-clotting enzyme	
	Free	Immobilized
ΔG^*_{E-T} (Free energy for transition state formation) = $-RT \ln (k_{cat}/K_m)$		
$Q_{10} = \text{antilog } E = (E \times 10/RT^2)$, Where $E = E_a$ = activation energy		

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 Mg^{+2} , Ca^{+2} and Mn^{+2} 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 k_d (deactivation rate constant) and higher $t_{1/2}$ (half-life time). In addition, immobilization improved the quality of MCE by decreasing the activation energy (E_a) by 0.9- fold compared to the free enzyme. Moreover, the E_{ad} (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

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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.

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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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