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Thermal Destabilization of *Rhizomucor miehei* Rennet with Aldehyde Dextran Sulfate: Purification, Bioconjugation and Milk-Clotting Activities

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Abstract High thermal stability of *Rhizomucor miehei* Rennet, which is a thermostable enzyme used in cheese production, causes undesired cases at elevated temperatures. This study aims to decrease the thermal stability of the *R. miehei* Rennet at high temperatures. To achieve this goal, bioconjugates of *R. miehei* Rennet with aldehyde derivative of dextran sulfate were synthesized in different molar ratios. Physico-chemical properties of bioconjugates were characterized with particle size analyzer and gel permeation chromatography (GPC) techniques. The enzyme and biopolymer were conjugated with medium efficiency. Milk-clotting activities of bioconjugates decreased drastically at high temperatures in all molar ratios, which reveals that covalent bioconjugation of the enzyme with aldehyde derivative of dextran sulfate caused a decrease in thermal resistance of this enzyme.

Keywords Bioconjugation · Dextran sulfate · Milk-clotting activity · *Rhizomucor miehei* Rennet · Purification · Thermal destabilization

Introduction

Enzymes have found a wide range of applications in miscellaneous industrial processes and products, from detergents and textile to food, paper, and plastic industries [1–6]. Cheese

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manufacture is one of these industries that enzymes, especially proteases, have been used since ancient times [7, 8]. High milk-clotting activity and low proteolytic activity are desired properties for a suitable protease used in cheese manufacturing [9, 10]. The term "rennet" is a general name for enzymes having milk-clotting activity, such as calf rennet, animal rennet, plant rennet, or microbial rennet [8]. Chymosin (calf rennet) extracted from the fourth stomach (abomasum) of the young calves has been used worldwide in cheese production for years [11]. Microbial rennets from *Rhizomucor miehei*, *Endothia parasitica*, and *Rhizomucor pusilllus* have been proven to be suitable for large-scale production [8]. *R. miehei* Rennet, which is used in cheese manufacture, is an aspartic protease which cleaves 105 (phenyl alanine)-106 (methionine) peptide bond in kappa-casein (milk protein) [12]. Microbial rennets have high thermal stability than calf rennets [8, 13]. *R. miehei* Rennet is the most thermostable enzyme of *R. miehei* at acidic pH values and is the most highly glycosylated one, having about 6 % carbohydrate content. It has been suggested by Yada et al. that this carbohydrate content helps prolong the active life of a glycoprotein by stabilizing conformation and by protecting the protein from proteolytic attack [10, 14]. High thermal stability of *R. miehei* Rennet (a) prevents the usage of by-products of food industry [11], for example, unutilized rennets exist in whey during the cheese production process [8] and (b) causes texture and flavor defects in the final product [9] which makes *R. miehei* Rennet undesired and disadvantageous in cheese manufacture. In addition to this, once whey which is by-product of food industry is used in baby foods, unutilized and active rennet may cause curd formation in milk solution. *R. miehei* Rennet should be thermolabile, because the enzyme has to be inactivated after curd formation by heat treatment (55 °C for 10 min). Low thermal stability is necessary to inactivate remaining enzymes in cheese whey using pasteurization procedure at high temperatures. Therefore, miscellaneous methods for chemical modification of *R. miehei* Rennet were developed to reduce its thermal resistance [9, 10, 15]. However, there are few papers about covalent conjugation and polycomplex formation of *R. miehei* Rennet with polymers to alter its thermostability [16, 17].

Covalent conjugation of enzymes with natural biopolymer materials, such as dextran and chitosan, has been studied for addition of novel desired characteristics to enzymes. Dextrans are easily water soluble, biodegradable, and biocompatible polysaccharides containing repeating glucose units linked with each other from α-1,6 and variable positions of α-1,2, α-1,3, and α-1,4 [18–20]. Dextran sulfate (DS) is the polyanionic derivative of dextran, which has double sulfate groups in each glucose unit, located at C2 and C4 of the glucose. DS adversely affects the enzymatic activity. It was shown that dextran sulfate decreases the activity of enzymes [21, 22]. Dextran or dextran sulfate biopolymers can be covalently conjugated to proteins via Schiff base formation with aldehyde groups in dextran/dextran sulfate chains and amine groups of protein [21, 23–25]. Aldehyde derivative of dextran or dextran sulfate may be easily prepared by oxidation with sodium periodate.

Milk-clotting activity (MCA) of *R. miehei* Rennet can be altered by modification of lysine residues on the enzyme surface [26]. Thermal stability of the *R. miehei* Rennet may be reduced by bioconjugation of *R. miehei* Rennet with aldehyde derivative of dextran sulfate (A-DS) through amine groups on the surface of enzyme. In the study, covalently bioconjugated *R. miehei* Rennet with A-DS was carried out, for the first time, in various molar ratios of components to reduce thermal stability of *R. miehei* Rennet to prevent undesired cases at high temperatures. Then, milk-clotting activities of *R. miehei* Rennet and its bioconjugates with aldehyde dextran sulfate were investigated at different temperatures and pH 5.0 to understand the effect of bio conjugation to the thermal stability of *R. miehei* Rennet.

Experiment

Materials and Methods

Rennet from *R. miehei* type II (Mw = 40 kDa) was purchased from Sigma. Skim milk powder was supplied by Pinar Co. (İzmir, Turkey). Calcium chloride (Riedel-de Haen), dextran sulfate (Mw = 100 kDa; Fluka), Sephadex G-50 (Fluka), Sephadex G-100 (Fluka), dialysis cellulose membrane (Sigma), $NaH_2PO_4.2H_2O$ (Riedel-de Haen), $Na_2HPO_4.7H_2O$ (Fluka), acetic acid (Fluka), sodium hydroxide (Riedel-de Haen), sodium periodate (Fluka), hydrochloric acid (Merck), and sodium borohydride (Merck) were used as received. Other reagents were of analytical grade.

Samples were lyophilized using Telstar Cryodos freeze-dryer system. Gel permeation chromatography (GPC) analyses of commercial and purified *R. miehei* Rennet and bioconjugates were acquired using Viscotek TDA 302 GPC with refractive index (RI) and UV (at 280 nm) detectors. Flow rate was adjusted to 1.0 mL/min. With Viscotek GPCmax pump system, Shimadzu Shim-Pack Diol 300 was used as column. Mobile phase was 0.100 M sodium phosphate buffer solution (PBS) at pH 7.0. OmniSEC 3.1 software was used for acquisition of chromatograms. All samples were filtered with 0.2-μm regenerated cellulose syringe filters. Hydrodynamic sizes of *R. miehei* Rennet and its bioconjugates were analyzed using a Malvern Zetasizer Nano ZS particle size analyzer. Enzymes and conjugates were purified with Bio-Rad protein purification system (model 2110 fraction collector) and concentrated with Millipore-stirred ultrafiltration cell (cellulose acetate ultrafiltration membrane; MWCO 10 kDa). UV-Vis spectrometry measurements were acquired with Shimadzu UV 1700 UV-Visible spectrophotometer. GFL 1086 shaking water bath was used to adjust temperature in enzyme activity studies.

Purification of *R. miehei* Rennet

R. miehei Rennet (Fluka; protein content 9.8 %), 2.0 g was dissolved in 10 mL 0.100 M sodium phosphate buffer (pH 7.0). *R. miehei* Rennet was purified through preparative gel filtration chromatography using Sephadex G–50 column (2.5 × 40 cm) at +4 °C with Bio-Rad protein purification system. Sodium phosphate buffer (0.1 M), was used at pH 7.0 as mobile phase. Eluates were collected as 5-mL fractions using Bio-Rad UV monitor at 280 nm in the fraction collector through 3 h. Tubes belonging to separate peaks were gathered together in separate flasks. Eluates of each peak were concentrated by using ultrafiltration membrane (cellulose acetate; cutoff 10 kDa) at +4 °C under nitrogen gas (10 mbar). Protein contents of concentrated solutions were determined with UV-Visible spectrophotometer according to Bradford method in our previous study [27].

Preparation of Aldehyde Derivative of Dextran Sulfate

A-DS was prepared according to Betancor et al. [28]. DS was oxidized with sodium periodate to obtain functional aldehyde groups for bio conjugation reaction with *R. miehei* Rennet. DS, 3.33 g was dissolved in 100 mL distilled water. Then, 8 g of sodium periodate was added to this solution to oxidize DS. This concentration of sodium periodates permits full oxidation of dextran sulfate. Mixture was incubated to react at room temperature in the dark for 24 h. The solution was dialyzed against distilled water to eliminate formaldehyde which is the by-product

of oxidation reaction. Then, aldehyde dextran sulfate solution was lyophilized. Lyophilized aldehyde dextran sulfate was used in bioconjugation reactions with purified *R. miehei* Rennet.

Bioconjugation of *R. miehei* Rennet with Aldehyde Derivative of Dextran Sulfate

Purified *R. miehei* Rennet was covalently conjugated with A-DS at different molar ratios ($n_{Rhizomucor\ miehei\ \text{Rennet}}/n_{\text{A-DS}}$ = 20/1, 10/1, 5/1, 1/1, 1/5, 1/10, 1/20). In all molar ratios of components, final concentration of protein was kept constant to 2 mg/mL. Two milliliter of enzyme solution containing 5 mg/mL of *R. miehei* Rennet in 0.1 M sodium phosphate buffer solution at pH 7.0 was incubated with 3 mL A-DS solution (A-DS concentration changes according to molar ratios, $n_{Rhizomucor\ miehei\ \text{Rennet}}/n_{\text{A-DS}}$) for 16 h at 25 °C and in slightly (80 rpm) shaking water bath. This reaction was stopped with sodium borohydride addition and pH was adjusted to 7.0. Then, pH of the solution was increased to 8.5 by adding 7.0 mL of 0.1 M sodium bicarbonate at +4 °C and remaining aldehyde groups, and the imine (–C = N–) bonds were reduced with 1 mg/mL of sodium borohydride addition. The solution was stirred for 15 min at +4 °C, and then, 1 mg/mL of sodium borohydride was added again and solution was stirred for 15 min at +4 °C. Then, pH of the solution was adjusted to 7.0.

To remove aggregates or impurities, *R. miehei* Rennet/A-DS conjugates were purified with preparative gel filtration chromatography technique (Sephadex G-100, 1.5 × 40 cm) at +4 °C. Sodium phosphate buffer (0.1 M) was used at pH 7.0 as a mobile phase in gel filtration column.

Milk-Clotting Activities

Milk samples were prepared freshly from skim milk according to our previous study [28]. 10 mM $CaCl_2$, was added to skim milk samples. One international unit is defined as the amount of enzyme which clots 10 mL of milk solution containing 1-g skim milk powder and 0.0173 g $CaCl_2$ in 1 min at 35 °C [29]. Skim milk (10 %) solution was prepared using different buffers for different pHs by adding 10 mM $CaCl_2$. 0.05 M Sodium acetate buffer, was used to prepare milk samples at pH 3.0, 4.0, and 5.0. 0.1 M Sodium phosphate buffer solution, was used to prepare milk samples at pH 6.0, 7.0, and 8.0. Precise pH values of milk samples were adjusted by slow addition of 1.0 M HCl or 1.0 M NaOH. Milk samples were left to rest at room temperature in a dark place for 30 min before using. Milk-clotting time of *R. miehei* Rennet and bioconjugates were measured using 10 mL of milk sample in a 25 mL beaker placed in a water bath at different temperatures and pHs. After the addition of 1 mL of *R. miehei* Rennet solution (0.04 mg/mL) to the milk, the coagulation time was measured with the visual method in our previous work [30]. The coagulation time was defined as the time passed until the first presence of graininess in the moving film of milk on the surface of the glass walls [31]. MCAs of *R. miehei* Rennet and its bioconjugates were determined according to Wu et al. [32].

Effect of pH on MCA of *R. miehei* Rennet and bioconjugates was investigated by measuring MCA at different pHs using different buffer solutions as mentioned above. All milk samples were equilibrated at 35 °C, and the coagulation times were investigated at pH values between 3.0 and 8.0 by adding 1 mL enzyme solution (0.04 mg/mL). Milk-clotting activity was not calculated at pH 3.0 because milk clotted spontaneously without enzyme addition at pH 3.0.

Effect of temperature on MCA of *R. miehei* Rennet and bioconjugates was investigated by measuring MCA at temperatures over the range of 25 to 65 °C and at pH 5.0. 1 mL conjugate (0.04 mg protein/mL), was used in all experiments.

Purified *R. miehei* Rennet and bioconjugates were incubated at different times (0, 5, 15, 30, 60 min) to determine their thermal stability or thermal destabilization at different temperatures (25 to 65 °C). Residual milk-clotting activities of purified *R. miehei* Rennet and bioconjugates were measured after heat treatment at different temperatures at pH 5.0.

All experiments were conducted with three replicates. Standard deviation was ≤3 %.

Results and Discussion

Purification of *R. miehei* Rennet and Thermal Stability of Purified *R. miehei* Rennet

Firstly, commercial *R. miehei* Rennet was purified because commercial *R. miehei* Rennet may contain impurities and unspecific proteases. Two peaks were observed after purification of *R. miehei* Rennet through preparative gel filtration chromatography at +4 °C in protein purification system (Supplementary material 1). Although MCA was observed in both peaks, MCA of first pool (57.7 IU/mg) was much higher than the second peak pool (1.5 IU/mg). Therefore, first pool was used in bioconjugation reactions. MCA of purified *R. miehei* Rennet (57.7 IU/mg) was found higher than MCA of unpurified *R. miehei* Rennet (25.9 IU/mg) at pH 5.0 and 35 °C. First-pool enzyme solution was concentrated with cellulose acetate ultrafiltration membrane and lyophilized. MCA of lyophilized *R. miehei* Rennet was low (40.5 IU/mg) in comparison with the concentrated enzyme at pH 5.0 and 35 °C. In further experiments, this concentrated enzyme was used.

Purity of *R. miehei* Rennet before and after purification was compared using GPC technique. GPC separates macromolecules according to their size in their native structures. One of the main methods to analyze protein purity is SDS-PAGE, but this method denatures proteins. Therefore, quaternary structures and aggregates of the proteins may not be observed. GPC chromatograms clearly show aggregates and multimers of proteins. GPC chromatograms of commercial and purified *R. miehei* Rennet are given in Fig. 1. It is clear from both RI and UV chromatograms that most of the aggregates and other impurities were removed successfully with preparative gel filtration chromatography by using Sephadex G-50 column.

In Fig. 1, red line was commercial *R. miehei* Rennet. Black line that was obtained from first-pool enzyme solution after purification of *R. miehei* Rennet as shown in Supplementary material 1 shows gel filtration chromatogram of commercial *R. miehei* Rennet acquired from Bio-Rad protein purification system (UV detector, printer, +4 °C, 1.0 range, 6 cm/h, 5 mL/tube).

Three peaks were existed in red line; however, one peak was observed in black line which is the purified *R. miehei* Rennet in Fig. 1. Obtained one peak supported the purification of the enzyme. Residual milk-clotting activities of commercial and purified *R. miehei* Rennet were shown in Fig. 2. Thermal stability of commercial and purified *R. miehei* Rennet was determined after treatment for 30- and 60-min incubation times at different temperatures and pH 5.0.

Figure 2 shows the comparison of MCA of purified and commercial *R. miehei* Rennet. MCA of purified enzyme is much higher than the commercial one at temperatures between 35 °C and 50 °C temperatures. Above 50 °C, MCAs of both enzymes decreased drastically. Results

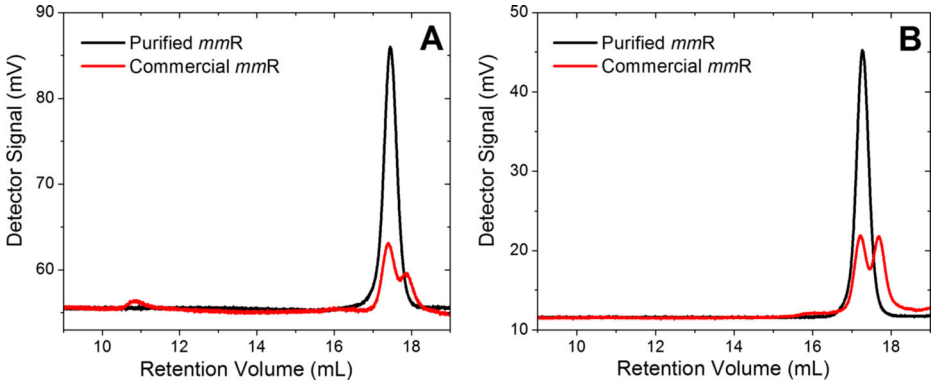


Fig. 1 GPC chromatograms of purified and commercial *Rhizomucor miehei* Rennet acquired from RI (**A**) and UV (**B**) detectors

reveal that purification of *R. miehei* Rennet from undesired impurities, such as carbohydrate moieties, caused an increase in its MCA below 55 °C. In addition, purified and unpurified enzymes did not show MCA or exhibit very low MCA at 60 °C after 30 min, respectively. MCA of commercial *R. miehei* Rennet still existed at 60 °C after 60 min of incubation in our study. Wu et al. (2013) studied the thermal stability of milk-clotting enzyme produced by *Bacillus subtilis* (natto) Takahashi [32]. Their enzyme indicated thermal stability at 40 and 50 °C for 120 min. Preetha (1997) found highest residual milk-clotting activity of purifying the milk-clotting protease from *R. miehei* at 35 °C for 30 min [33]. Residual milk-clotting activity decreased above 35 °C. He et al. (2011) studied purification and characterization of the milk-clotting enzyme produced by *Bacillus amyloliquefaciens* D4 and found optimum temperature at 65 °C [34]. He et al. found thermal stability of the enzyme decreasing above 35 °C for 60-min milk-clotting activity of purified *R. miehei* Rennet that has been shown in Fig. 3 at different pHs and temperatures. MCA of purified *R. miehei* Rennet was high at acidic pHs (4.0, 5.0, and 6.0). Wu et al. (2013) found optimum pH and temperature for purified enzyme at pH 6.0 and 60 °C, respectively [32].

MCA of the enzyme increased at high temperatures and acidic pHs. MCA of *R. miehei* Rennet was drastically reduced at pH 7.0 and pH 8.0. These results show that the purified

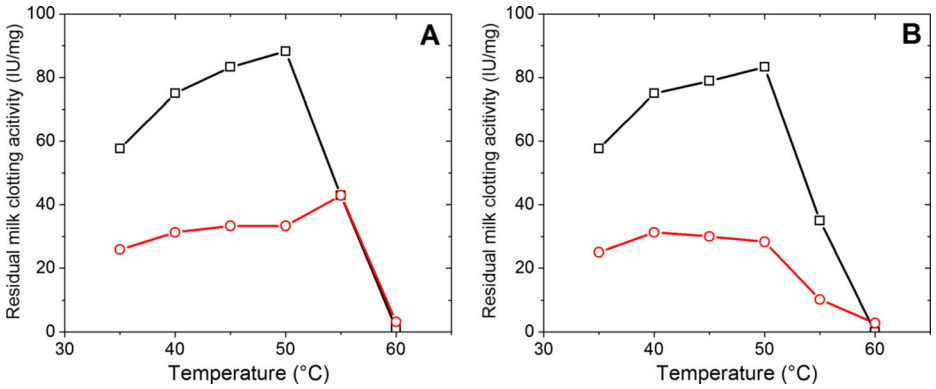


Fig. 2 Residual milk-clotting activities of purified *Rhizomucor miehei* Rennet (*black square*) and commercial *R. miehei* Rennet (*red circle*) after treatment for 30 min (**A**) and after treatment for 60 min (**B**) at different temperatures and pH 5.0

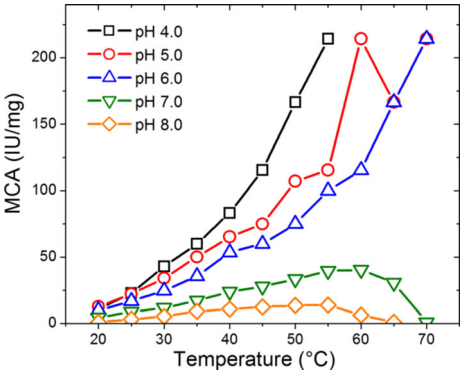


Fig. 3 Milk-clotting activities of purified *Rhizomucor miehei* Rennet at different temperatures and pHs

R. miehei Rennet displays high milk-clotting activity at acidic pH values and especially at pH 5.0 and pH 6.0 in Fig. 3. Table 1 gives a comparison of purification and optimum pH, temperature, and thermal stability of the enzyme with previous studies in the literature. Preetha (1997) used purified milk-clotting enzyme and found 35 °C for temperature stability of milk-clotting enzyme [33]. They found optimum milk-clotting activity at pH

Table 1 Comparison of purification methods and optimum conditions for microbial rennets with this study

Microorganism	Purification	Optimum pH and temperature for MCA	Thermal and pH stability	$CaCl_2$ (mM)	Ref
Bacillus amyloliquefaciens D4	Ammonium sulfate and ion-exchange chromatography	65 °C, pH 5.5	Thermal stability 35 °C, pH stability 6.5	25 mM	He et al. 2011 [34]
Thermomucor indicae-seudaticae N31	Centrifugation at 30,996 rpm for 20 min	pH 5.7, 70 °C	pH range 3.5–4.5 for 24 h	40 mM	He et al. 2011 [34]
Nocardiopsis sp.	Fractional precipitation with ammonium sulfate and DEAE-cellulose chromatography	55 °C	Thermal stability 35 °C for 90 min pH stability wide range 4.5–11.0	10 mM	Hermanson 2013 [35]
Rhizomucor miehei	Ion-exchange and affinity chromatographies	pH 5.6	Thermal stability 35 °C for 30 min at pH 5.6 (0.05 M sodium acetate buffer)	10 mM	Preetha and Boopathy 1997 [33]
R. miehei Rennet	Gel filtration Sephadex G-50 at pH 7.0 (0100 M, phos-phate buffer) and ultrafiltration with cellulose acetate membrane (cutoff 10 kDa)	50 °C, pH 5.0	Thermal stability 35–50 °C (wide range) through 60 min at pH 5.0 (0.05 M sodium acetate buffer)	10 mM	In this study

5.6. More results about optimum pH and temperature and thermal stability of purified rennets were summarized in Table 1.

Synthesis of *R. miehei* Rennet/A-DS bioconjugates

In the first step of the synthesis, DS was oxidized to its aldehyde derivative with sodium periodate, which is the simplest method for converting hydroxyl groups on sugar rings into amine-reactive aldehydes. Oxidation of sugar rings with periodate results in sugar ring opening, and many aldehyde groups are formed along polysaccharide chains [35]. Bioconjugation of *R. miehei* Rennet with aldehyde dextran sulfate in a wide range of the ratio of components was accomplished using Schiff base formation between amine groups of enzyme and aldehyde groups of oxidized DS.

Following oxidation, A-DS was conjugated with *R. miehei* Rennet; GPC chromatograms of A-DS, *R. miehei* Rennet, and A-DS/*R. miehei* Rennet conjugates are given in Fig. 4. Purified *R. miehei* Rennet is eluted at 17.2 mL as a single and narrow peak in both of RI and UV chromatograms. A-DS elutes as a broad peak in RI chromatogram which indicates the polydisperse nature of the polymer. In UV chromatogram, A-DS gives no signal due to its very low absorption at 280 nm. After conjugation, peaks belonging to each component can be clearly seen in RI chromatograms. The peak area of A-DS increases with the increase in molar ratio of components. Elution volume of the enzyme did not change significantly, but peak shape of the A-DS changed according to its concentration and conjugation. In UV chromatograms of all conjugates, a new peak elutes earlier than enzyme (~16 mL). In addition, a broad peak is seen in UV chromatogram of the conjugate with the ratio of 1/10, which is one of the conjugates with highest A-DS concentrations. UV chromatograms show that higher-molecular-weight compounds are formed, which clearly indicates that *R. miehei* Rennet and A-DS are covalently conjugated with low conjugation yield. GPC chromatograms of the bioconjugates with other ratios ($n_{Rhizomucor\ miehei\ \text{Rennet}}/n_{\text{A-DS}}$ = 10/1, 1/1, 1/5, 1/20) of components are not given in the Fig. 4, since similar chromatograms were obtained.

Hydrodynamic size distributions of A-DS, *R. miehei* Rennet, and A-DS/*R. miehei* Rennet bioconjugates were determined using Malvern Zetasizer Nano ZS particle size analyzer. Hydrodynamic size distributions are given as volume dependent in Fig. 5. The hydrodynamic diameter of *R. miehei* Rennet is 5.5 nm. A-DS has a bimodal distribution and diameter of the peak with largest area (96.8 %) is 11.7 nm. Area of other peak of A-DS is 3.2 % and size of this peak is

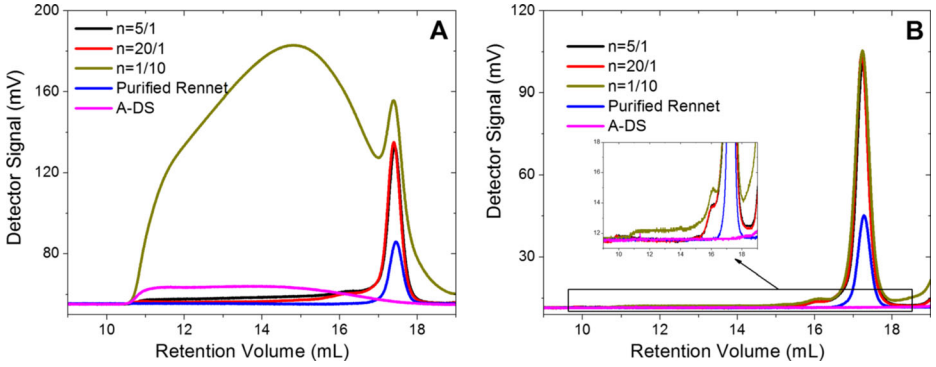


Fig. 4 GPC chromatograms of purified rennet, A-DS, and bioconjugates of different protein/biopolymer ratios acquired from RI (**A**) and UV (**B**) detectors

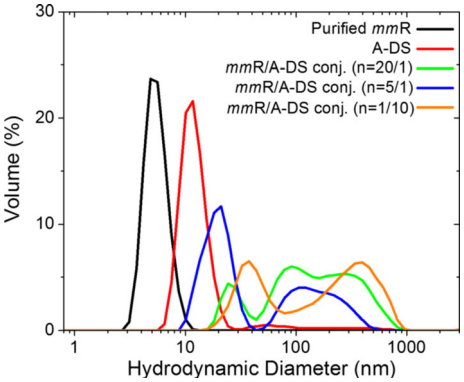


Fig. 5 Hydrodynamic size distributions (by volume) of *Rhizomucor miehei* Rennet, A-DS, and bioconjugates with molar ratios of 20/1, 5/1, and 1/10

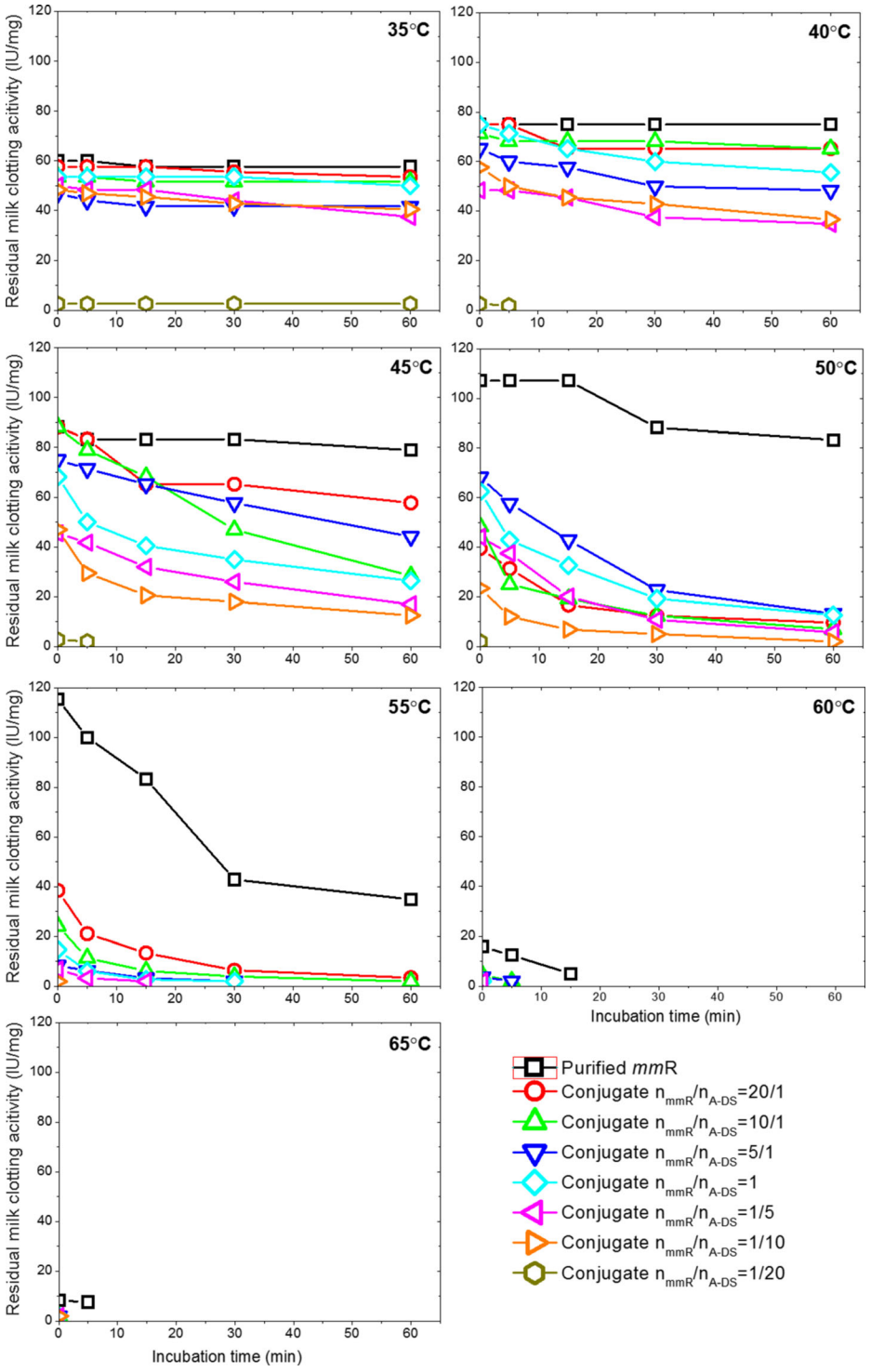
84.3 nm. Size distributions of all conjugates are also bimodal. All peaks of conjugates are larger than *R. miehei* Rennet and peak at 11.7 nm of A-DS. Diameters of second peaks of all conjugates are larger than both *R. miehei* Rennet and A-DS. Size distributions of the conjugates with other ratios of components ($n_{Rhizomucor\ miehei\ \text{Rennet}}/n_{\text{A-DS}}$ = 10/1, 1/1, 1/5, 1/20) are not given in the figure since similar size distributions were obtained.

In Fig. 5, these results show that larger macromolecules than individual *R. miehei* Rennet and A-DS were formed; this reveals the successful bioconjugation of *R. miehei* Rennet and A-DS. On the contrary to GPC results, free *R. miehei* Rennet peaks were not observed in Zetasizer results. Hydrodynamic size distributions given in Fig. 5 are volume dependent. Since *R. miehei* Rennet/A-DS conjugates have larger sizes than both of *R. miehei* Rennet and A-DS, they have higher volume and light-scattering signals. This can be the reason that free *R. miehei* Rennet was not observed in size distribution graphics. According to the results, direct relation between size of the conjugate and ratio of components was not observed. GPC and Zetasizer reveal the formation of *R. miehei* Rennet/A-DS bioconjugates. GPC results indicate the medium yield of bioconjugation in Fig. 4. Bioconjugation reaction was successfully revealed by results of GPC and particle size analysis.

Milk-clotting activities of bioconjugates (*R. miehei* Rennet/A-DS)

Milk-clotting enzymes are used for cheese making in food industry. *R. miehei* Rennet is thermostable milk-clotting enzyme. This study aims to decrease thermal stability of *R. miehei* Rennet at high temperatures, since it is necessary to heat whey at 62.2 °C for 30 min at pH 6.6 to inactivate (at least 95 %) all coagulants in cheese production [8]. For this purpose, purified enzyme was covalently bioconjugated with A-DS in different molar ratios and milk-clotting activities of all conjugates were investigated at different temperatures and at pH 5.0.

Before bioconjugation of *R. miehei* Rennet and A-DS, *R. miehei* Rennet and DS were physically mixed in same molar ratios of conjugates ($n_{Rhizomucor\ miehei\ \text{Rennet}}/n_{\text{DS}}$ = 20/1, 10/1, 5/1, 1/1, 1/5, 1/10, 1/20) to understand the effect of free DS chains on *R. miehei* Rennet activity. It was observed that physical mixtures of *R. miehei* Rennet with DS had similar activity with purified unconjugated *R. miehei* Rennet. At pH 5.0, both *R. miehei* Rennet (pI = 4.1) and DS are negatively charged which prevents interaction between them, and enzyme activity remains unchanged.


35°C
40°C
45°C
50°C
55°C
60°C
65°C
Residual milk clotting acitivity (IU/mg)
Incubation time (min)
Purified mmR
Conjugate n_mmR/n_A-DS=20/1
Conjugate n_mmR/n_A-DS=10/1
Conjugate n_mmR/n_A-DS=5/1
Conjugate n_mmR/n_A-DS=1
Conjugate n_mmR/n_A-DS=1/5
Conjugate n_mmR/n_A-DS=1/10
Conjugate n_mmR/n_A-DS=1/20

◀ **Fig. 6** Residual milk-clotting activities purified *Rhizomucor miehei* Rennet and *Rhizomucor miehei* Rennet/A-DS biioonjugates at different molar ratios of components (20/1, 10/1, 5/1, 1/1, 1/5, 1/10, and 1/20) at different temperatures (35, 40, 45, 50, 55, 60, and 65 °C) and after treatment various incubation times (0, 5, 15, 30, and 60 min) at pH 5.0

Bioconjugation of *R. miehei* Rennet with high negatively charged A-DS aims to preserve its high activity at cheese-manufacturing temperature (35 °C) and also inhibits all milk-clotting activity above 50 °C. Residual milk-clotting activities of purified *R. miehei* Rennet and *R. miehei* Rennet-A-DS bioconjugates were shown in Fig. 6 after treatment with different incubation times (for 5, 15, 30, and 60 min) at pH 5.0.

Purified *R. miehei* Rennet showed high milk-clotting activity in all temperatures, but at 60 and 65 °C, milk-clotting activity was not observed after treatment 30-min incubation. In Fig. 6, most prominent change in enzyme activity was observed in the ratio of 1/20. In this ratio, milk-clotting activity of the enzyme drastically decreased and it almost had no activity in all temperature range. This may be the result of aggregation of *R. miehei* Rennet with high concentration of A-DS or with other conjugate molecules in solution. This ratio of bioconjugate is not a proper candidate for use in cheese production.

In other ratios of components, a significant decrease in *R. miehei* Rennet activity was observed at temperatures above 50 °C in comparison with purified *R. miehei* Rennet. Most promising results in all these ratios of conjugates were obtained in the ratio of 1/10. In this ratio, activity of enzyme reduced 84 and 98 % compared with purified enzyme at 45 and 50 °C and shows high activity in cheese production temperature. In addition, milk-clotting activity drastically decreased and was not observed at high temperatures preventing of undesired cases at high temperatures (55–60–65 °C), such as curd formation when whey which is by-product of cheese manufacturing is used in baby foods because of retained milk-clotting activity of the enzyme. In addition to this, bioconjugates of *R. miehei* Rennet indicated high milk-clotting activity at 35 °C temperature.

Thermal Stability

Low thermal stability is necessary to inactivate remaining enzymes in cheese whey using pasteurization procedure at high temperatures. The effect of temperature on the stability of commercial and purified *R. miehei* Rennet is shown in Fig. 2 after treatment for 30 and 60 min at different temperatures. After purification, MCA of purified enzyme was higher at temperatures between 35 and 50 °C than the commercial enzyme. Thermal stability of the enzyme was slightly decreased with purification of *R. miehei* Rennet at high temperatures of 55 and 60 °C and at pH 5.0 in this study. Thermal stability studies of purified rennets in the literature were summarized in Table 1. Our results were compared with other studies (Table 1). In this study, the purified enzyme did not show milk-clotting activity at 65 °C and pH 5.0 in Fig. 6. Residual milk-clotting activity of purified enzyme and bioconjugates was performed to determine thermal stability after treatment for 60 min at pH 5.0.

Supplementary material 2 shows thermal stability of the purified *R. miehei* Rennet and bioconjugates after heat treatment at different temperatures for 60 min at pH 5.0. Bioconjugates shows high milk-clotting activity and thermal stability at 35 and 40 °C. All bioconjugates showed very low residual activity at 45 and 50 °C. In addition to this, all conjugates were inactivated above 50 °C for 60 min.

Conclusion

In the study, modification of *R. miehei* Rennet was firstly made with a biopolymer by covalent conjugation. Effect of pH, temperature, and incubation time on milk-clotting activity of the *Rhizomucor miehei* Rennet and bioconjugates were investigated. The purified enzyme displays optimum milk-clotting activity at pH 5.0 and 50 °C temperature. Bioconjugation of *R. miehei* Rennet with aldehyde dextran sulfate drastically reduced the thermal stability of the enzyme at high temperatures. Inactivation of the enzyme-biopolymer bioconjugates at high temperatures makes the enzyme suitable for use in cheese and whey production employing pasteurization procedure. Bioconjugation caused total inactivation of milk-clotting activity of *R. miehei* Rennet at above 50 °C temperatures.

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