FRACTIONATION OF TRANSGLUTAMINASE PRODUCED BY *STREPTOMYCES MOBARAENSIS* ATCC 27441

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Abstract

Microbial transglutaminase (mTG) can catalyze covalent bonds between proteins, a property used in the food industry to restructure meat, improve the texture of dairy products, and extend the shelf life of foods. In our study, mTG was obtained using Streptomyces mobaraensis ATCC 27441 strain and fractionation using various ion exchange resins. The enzymatic activity was monitored using a spectrophotometric method. The results showed that the use of the Fractogel EMD SO_3^- resin led to the highest yield in the fractionation process (84.23%), recovering an enzymatic activity of 1613.83 U. Using Relisorb, a total enzymatic activity of 882.07 U and a yield of 46.04% was evidenced. In the case of Amberlite CG-50, a total activity of 869.558 U was achieved, with a final yield of 45.38%. The lowest final yield was recorded for Resindion SP 825L resin - 5.08%, with an enzymatic activity of 97.420 U. The results indicate that Fractogel EMD SO_3^- is the most efficient resin for mTG fractionation, providing a robust approach for obtaining a high-purity enzyme suitable for industrial applications.

Key words: *transglutaminase, Streptomyces mobaraensis, fractionation, ion exchange chromatography*

INTRODUCTION

Enzymes purification is an essential step for their use in biotechnology, pharmaceutical and food industry, as crude extracts obtained from microbial sources often contain impurities that reduce stability, activity and catalytic specificity. The aim of the purification process is to obtain enzymes with high yield, optimal catalytic activity and an advanced degree of purity, to ensure industrial performance and scientific reproducibility. Established methods include precipitation, chromatography and electrophoresis, each with advantages and limitations. Precipitation, usually with salts or organic solvents (ethanol, ammonium sulfate), allows the rapid separation of proteins by modifying their solubility and collecting them by centrifugation. Chromatography (ion exchange, gel filtration or affinity) ensures the separation of proteins based on their interactions with the column matrix. Electrophoresis, especially SDS-PAGE, is used complementary, both for the analysis of molecular mass and purity, and for the effective separation of protein fractions [1].

Numerous studies aimed at applying these methods for the isolation of microbial transglutaminases. Cui *et al* (2007) described the purification of the enzyme from *Streptomyces hygroscopicus* using a classic sequence of steps: fermentation for 42 h, centrifugation to remove biomass, precipitation with cold ethanol up to 70% (which led to the recovery of ~85% of the enzyme and a doubling of the specific activity), followed by chromatography on CM-cellulose and separation on Sephadex G-75. Finally, a 25-fold purification and a

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yield of 57.7% were obtained, but the stability was negatively influenced by high ethanol concentrations and high temperatures [2].

Streptomyces mobaraensis DSM 40587 cultivated for 7 days in a medium rich in salts and polypeptone was also used to obtain mTG. After 96 h of fermentation, the culture liquid was subjected to ultrafiltration and purified by chromatography, leading to a final specific activity of 17.20 U/mg and a high purity enzyme. Biochemical analyses showed optimal activity at pH 6.0 and 55 °C, with stability between pH 5.0-10.0 at 40 °C. Kinetic parameters revealed a Km value of 40.47 mmol/L and a maximum Vmax of 44.44 U/mg. The activity was strongly inhibited by Zn^{2+} and Cu^{2+}, but was not affected by Na^{+}, K^{+}, Ca^{2+} or Ba^{2+} ions, which underlines the industrial potential of the enzyme [3].

Further, the process was optimized for *Streptomyces mobaraensis*. Thus, a two-step purification: ethanol precipitation (50% w/v) and combined chromatography (cation exchange on SP Sepharose and hydrophobic interactions on phenyl-Sepharose) was employed, resulting in a yield of 44% and a specific activity of 39.2 U/mg. The N-terminal sequence analysis confirmed the presence of a zymogen-specific signal peptide. From a kinetic point of view, the reaction followed a "ping-pong" mechanism, with Km values of 23.12 mM for the substrate N-α-CBZ-Gln-Gly and 1.37 mM for hydroxylamine hydrochloride. The enzyme was stable between pH 5 - 10 and optimally active at pH 6.0 and 45 - 50 °C, but severely inhibited by Zn^{2+}, Cu^{2+} and Fe^{3+}, limiting its use in industrial environments rich in these cations [4].

The classical methods were complemented by patented developments, with the aim of streamlining costs and increasing the process yield. In patent CN105754892A, the transglutaminase from *Streptomyces mobaraensis* was isolated by a simplified scheme: cold alcohol precipitation, centrifugation, anion exchange chromatography on Q Sepharose, desalting and lyophilization. The procedure provided an enzyme with 90% purity, specific activity of 25-30 U/mg, low endotoxin content (0.02 EU/mL) and an overall yield of 70%. The main advantage of this method lies in the combination of simplicity, cost-effectiveness and industrial scalability, which recommends it for large-scale production, including in the food and pharmaceutical industries [5].

The comparative analysis of purification strategies highlights both the evolution of the methods and the remaining challenges: sensitivity to metal cations, optimization of culture conditions and separation steps. The microbial transglutaminases obtained by these methods show good biochemical characteristics, therefore the improvement of purification processes is an essential step for their exploitation on an industrial scale. Therefore, in our study, we compare the efficiency of three different methods used for the separation of the mycelium from the culture liquid, as well as four types of ion exchange resins used for fractionation in the case of transglutaminase obtained in a fermentation process based on a *Streptomyces mobaraensis* strain.

MATERIAL AND METHOD

Microorganism and culture conditions

Streptomyces mobaraensis ATCC 27441 strain was selected to produce transglutaminase using a fermentation medium based on the formula presented by Alves Macedo *et al.*, 2007 [6]. The cultivation conditions were set as follows: temperature - 28 °C, pH uncontrolled (free), and the dissolved oxygen level adjusted to 30%. To achieve and maintain this value, the fermenter control system was operated in a "cascade" mode, by automatically adjusting the agitation speed (180 ÷ 250 RPM) and the aeration flow rate (0.5 ÷ 1.2 vvm). The culture liquid was obtained by

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the conventional method of submerged fermentation. Enzymatic activity was monitored throughout the entire process.

Fractionation of mTG

Three different methods were used to separate mycelium from the culture liquid: centrifugation (8000 RPM for 20 minutes), vacuum filtration (using Whatmann filter paper no. 2 and a vacuum pump) and microfiltration (using 0.1-10 µm pore sizes membranes).

Extraction and fractionation of transglutaminase was performed using ion exchange chromatography. Four types of ion exchange resins were used: Amberlite CG-50, Resindion SP 825L, Relisorb and Fractogel EMD SO_3^-. The resins were activated according to the manufacturer's instructions. Glass columns loaded with 12 mL of activated resin were used for the fractionation step. The centrifuged supernatant, with a known activity and a pH of 6.0 was absorbed onto the resin with a flow rate of 1 mL/minute, using a peristaltic pump. Subsequently, the resin was washed with monosodium phosphate buffer solution (pH 6.5) and different concentrations of sodium chloride (0.1 M, 0.2 M, 0.3 M and 0.4 M) were used for the gradient elution step. Fractions were collected and the enzymatic activity was determined.

For evaluating the efficiency of the transglutaminase fractionation process, the yield was calculated by relating the enzymatic activity obtained from mixing the set of fractions recovered from the elution process to the total initial enzymatic activity of the supernatant.

This ratio represents how effective the resin and fractionation process were in preserving and recovering the enzymatic activity after washing and elution steps. The final result was expressed as a percentage, indicating the proportion of the initial enzymatic activity that was recovered after fractionation.

Transglutaminase activity assay

Microbial transglutaminase activity (U/mL) was determined by a colorimetric procedure described by Folk and Cole (1966). N-carbobenzoxy-L-glutamyl-glycine was used as a specific substrate, and the resulted benzoic acid was determined spectrophotometrically [7]. Enzyme activity was calculated using the following formula:

$$A(U/mL) = \frac{\text{Sample abs.}}{\text{Standard abs.}} \times \text{Standard conc.} \times \text{Dilution} \times 0.04$$

where the sample absorbance (nm) is that read by the spectrophotometer, standard absorbance = 0.2774, and standard concentration = 2.5 mmol/L.

Statistical analysis

The results were analyzed using GraphPad Prism8 software. Pearson correlation was used to analyze the relation between incubation time and enzymatic activity. The results were considered significant for a P-value < 0.05.

RESULTS

Cultivation conditions were strictly monitored during the fermentation process carried out in a 20 L fermenter. The temperature was kept constant at 28 °C, and the pH was not controlled, evolving freely according to the metabolism of the microorganism. The dissolved oxygen was set at 30% and maintained by a cascade control system, which adjusted both the stirring speed and the aeration rate. Thus, the stirring speed varied between 180 and 250 RPM, and the aeration flow rate between 0.5 and 1.2 vvm, the values being automatically adapted to prevent oxygen limitation. Under these conditions, the biomass recorded a constant increase, with a progressive accumulation in the first 40 hours of cultivation, followed by a stabilization phase, specific to the transition to the stationary phase. The evolution of pH followed the metabolic trend characteristic

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of actinomycetes, with a slight acidification in the early phases, followed by a gradual increase with substrate consumption and accumulation of secondary metabolites.

It can be seen that in the first 12.5 hours, the enzymatic activity is zero (0 U/mL), most likely due to the fact that in this phase the cells adapt to the culture medium and activate their mechanisms necessary for growth, without producing a significant amount of mTG. From 12.5 to 36 hours, the enzymatic activity increased, reaching 4.1 U/mL (with a significant increase at 18 hours - 0.89 U/mL), indicating the beginning of the enzymatic production phase. Between 36 and 40 hours, the enzymatic activity increased rapidly, reaching a maximum of 8.15 U/mL at 40 hours. The sudden increase is probably related to the achievement of optimal culture and metabolic conditions that favor the synthesis of transglutaminase. After 40 hours of incubation, enzymatic activity decreased to 7.53 U/mL, most likely due to the unavailability of essential nutrients in the culture medium (Fig. 1).

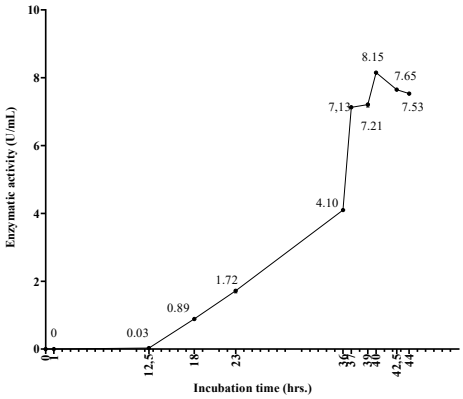

Fig. 1. Dynamic of transglutaminase activity over a period of 44 hours of incubation

Concerning the methods used to separate mycelium from the culture liquid, centrifugation was the most efficient, with a yield of 97.94%, compared to microfiltration (36.14%) and vacuum filtration (77.90%) - Fig. 2.

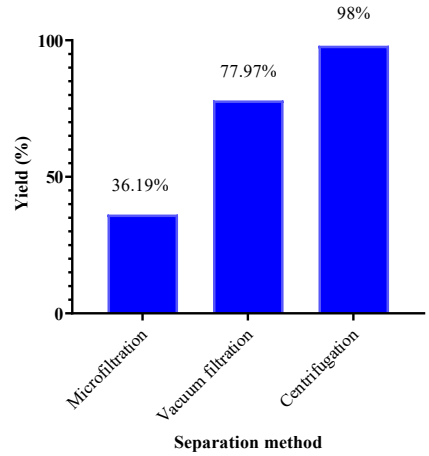

Fig. 2. The yield of different separation methods

The steps of the fractionation process were the same for all types of investigated resins (Fig. 3). In the case of Amberlite CG-50 resin, during the absorption step, the enzyme activity decreased significantly to 1.2 U/mL, with a total activity of 239.5 U, indicating that a significant part of the enzyme was bound to the resin. Washing was successful in removing impurities, with a recorded enzyme activity of 1.71 U/mL. During the five elution steps, the enzyme activity varied, with a maximum of 3.89 U/mL in the second elution, and a minimum of 0.97 U/mL in the fifth elution. The overall process yield was 45.38%, reflecting the moderate efficiency of Amberlite CG-50 resin in the fractionation of transglutaminase. In the case of Resindion SP 825L resin, after the first absorption the enzymatic activity decreased to 2.51 U/mL, and after the second absorption, it increased slightly to 3.14 U/mL. Washing with buffer maintained an enzymatic activity of 1.71 U/mL. Low enzymatic activities were recorded in the elution steps, with a maximum of 0.42 U/mL in the first elution and a minimum of 0.23 U/mL in the third

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elution. The final yield was very low, only 5.08%, indicating a low efficiency of Resindion SP 825L resin for this fractionation process. The use of Relisorb resin started with the same initial conditions, but after the first absorption, the enzymatic activity was 0.63 U/mL, and after the second absorption it increased to 2.29 U/mL. The washing allowed the recording of an enzymatic activity of 3.03 U/mL. During the elutions, the enzymatic activity reached a maximum of 5.99 U/mL in the first elution and gradually decreased to 0.37 U/mL in the fifth elution step. The total yield was 46.04%, suggesting that the Relisorb resin has a similar efficiency to Amberlite CG-50 in the fractionation of transglutaminase, with a slight improvement. The best results were recorded in this study for Fractogel EMD SO_3^- resin. After absorption, we recorded an enzymatic activity of 0.72 U/mL.

Fig. 3. Steps used for transglutaminase fractionation process

In the first wash, the enzymatic activity was 1.53 U/mL, and the second wash decreased the activity to 0.83 U/mL. The first elution step allowed recording a high enzymatic activity of 6.41 U/mL, and the second elution of 1.13 U/mL. The final yield of the process was 84.23%, indicating a high efficiency of the Fractogel EMD SO_3^- resin in fractionation of microbial transglutaminase (Fig. 4).

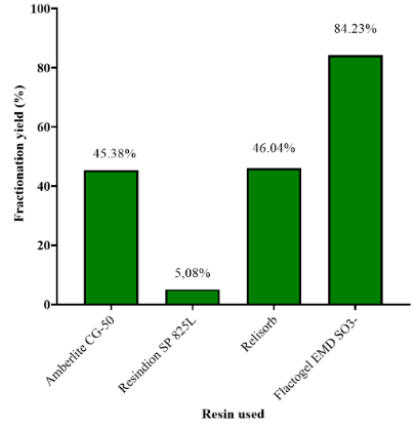

Fig. 4. Fractionation of transglutaminase using different ion exchange resins

DISCUSSIONS

Comparing these results with those reported in the literature, one may observe that the enzymatic activity of transglutaminase produced by *Streptomyces mobaraensis* ATCC 27441 is significantly higher in a much shorter time. While Téllez-Luis et al. (2004) reported an activity of 0.34 U/mL after 72 hours of biosynthesis on a medium containing sorghum hydrolysate [8], our results indicated an activity of 0.89 U/mL after only 18 hours, highlighting the high efficiency of the culture conditions used. Also, the enzymatic activity measured at 40 hours (8.15 U/mL) significantly exceeds the values obtained by Portilla-Rivera et al. (2009), who reported 0.46 U/mL using sugarcane molasses and glycerin [9], and by Guerra-Rodríguez and Vázquez (2013), who obtained 1.12 U/mL after 30 hours using potato enzymatic hydrolysates supplemented with yeast and corn extract [10]. These differences highlight the efficiency of the culture medium and fermentation conditions used

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in our experiment, which allowed not only a faster increase in enzymatic activity, but also the achievement of higher maximum values compared to those reported previously in the literature.

A very strong positive correlation was recorded between enzymatic activity and incubation time ($r = 0.9140$), meaning that the enzymatic activity increases significantly ($P = 0.0006$) with the growth of the culture.

The separation yields varied depending on the method used to separate the mycelium from the culture liquid. Microfiltration led to a significant loss of enzymatic activity (2.94 U/mL final compared to 8.15 U initial), resulting in a yield of only 36.14%. The decrease in enzymatic activity may be attributed to the retention or destruction of the enzyme at the level of microfiltration membrane. In addition, the volume of the final sample is significantly reduced, suggesting that a large part of the liquid did not pass through the filter. Regarding vacuum filtration, this separation technique maintained almost constant enzymatic activity (6.34 U/mL final compared to 8.15 U/mL initial), resulting in a yield of 77.90%. In this case, the final sample volume is reduced, but the enzymatic activity remains constant, suggesting that the enzyme was preserved in the aqueous phase. Centrifugation led to the concentration of the enzyme in the supernatant (7.98 U/mL final compared to 8.15 U/mL initial), resulting in a higher yield of 97.94%. Thus, we may presume that the increase of enzymatic activity is due to the concentration of transglutaminase in the supernatant obtained after mycelium sedimentation. The centrifugation process effectively separates cells and other solid particles from the liquid, keeping the transglutaminase in the aqueous phase.

Ion exchange resins are used to optimize the efficiency and fractionation yield in the microbial transglutaminase fractionation process. The appropriate choice of resin allows the removal of impurities and the maximum recovery of enzymatic activity, thus ensuring a high purity final product. In our study, each resin was tested using a similar protocol, which included absorption, washing and multiple elution steps with NaCl solutions of different concentrations. The superior efficiency of Fractogel EMD SO_3^- resin in the microbial transglutaminase fractionation process is mainly due to the presence of sulfonate ($–SO_3^-$) functional groups. These groups give the resin a strong cation exchange character, establishing strong electrostatic interactions with the positively charged enzyme molecules at the working pH. The transglutaminase was efficiently retained on the column, and subsequently, by controlled increase of the ionic strength of the elution buffer, fractional release of the enzyme was possible under reproducible conditions. This property ensured not only selective retention, but also a clear separation of the target protein from protein impurities, with minimal loss of activity. We may presume that the sulfonate groups were the determining element of the performance of Fractogel EMD SO_3^-, explaining the high yields and superior purity of the fractions obtained compared to other resins used.

CONCLUSIONS

The results obtained in this study demonstrate that *Streptomyces mobaraensis* ATCC 27441 constitutes an efficient source for the production of microbial transglutaminase.

Regarding the separation of the mycelium, centrifugation proved to be the most efficient method (97.94%), compared to vacuum filtration (77.90%) and microfiltration (36.14%), confirming the applicability of this technique in the rapid and efficient recovery of the enzyme from the culture liquid.

In the fractionation step, the use of ion exchange resins revealed significant differences in efficiency. Relisorb and

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Amberlite CG-50 led to moderate yields (46.04% and 45.38%, respectively), while Resindion SP 825L proved ineffective, with a yield of only 5.08%. In contrast, Fractogel EMD SO_3^- resin presented the best performances, with a yield of 84.23% and a total recovered activity of 1613.83 U, confirming its high affinity for microbial transglutaminase and its potential for industrial scale application.

Thus, this study makes significant contributions to the optimization of microbial transglutaminase fractionation processes, highlighting the resin used in ion exchange chromatography. The results support the integration of the developed method into an industrial setting, providing a scalable process with high yields and a final product with superior purity.

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