Evaluation of the Eversa® Transform 2.0 structure changes in pressurized R134a

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ABSTRACT

This study aimed to comprehensively examine the effects of pressurized 1,1,1,2-tetrafluoroethane (R134a) on Eversa® Transform 2.0 (ET2.0) immobilized based silica supports, specifically elucidating alterations in secondary structure. Immobilization of ET2.0 on Amine-glutaraldehyde-silica (AmG) were treated in different conditions of pressure (50-150 bar), temperature (30-50°C) exposure time (2-7 hours) in a variable volume high-pressure visualization cell. After the exposure period, a hydrolytic activity in olive oil of the AmG biocatalyst was carried out to verify the residual activity. ET2.0 immobilized were analyzed by using Fourier Transform Infrared (FTIR) spectroscopy self-deconvolution compared with the conformational change in ET2.0 without treatment and post-treatment in high-pressure. Using high pressure technologies provided an increase in the catalytic activity of the biocatalyst, reaching a residual activity of around 260% at 100 bar, 40°C for 4.5h. The best residual activity results generated biocatalysts with lower percentages of α-Helix and an increase in the β-Sheet region when compared to the control (enzyme without high pressure treatment). This behavior implicated the increased flexibility and mobility of the protein under pressure. This work offers new possibilities for using synthesized silica-based biocatalysts to promote pre-activation at high pressure and subsequent application in reactions of industrial interest.

Keywords: Enzymatic stability. Conformational change. Eversa® Transform 2.0. Immobilization. Based silica.

1 INTRODUCTION

High-pressure technologies can be valuable tools for modifying the structure and functional properties of various proteins and enzymes[1]. Despite this potential, the impact of these technologies on enzyme structure remains largely unexplored. Nevertheless, their application could significantly facilitate process scale-up, a crucial aspect in industrial product development. Additionally, they could enhance the viability of enzymatic routes[2].

The application of high-pressure technologies in enzymatic processes shows great potential to improve catalytic activity, owing to the greater stability of the biocatalyst, reduced diffusional effects, and increased mass transfer within the system[2,3]. However, few studies have investigated the possible structural modifications and activity of enzymes when exposed to this emerging technology, particularly in the context of synthesized immobilized biocatalysts.

A class of enzymes that demonstrates significant improvement in catalytic activity and stability when immobilized on a specific support and applied under normal pressure conditions is lipases. Belonging to the hydrolase class, lipases can perform reactions in both aqueous (hydrolysis) and organic (transesterification, esterification, among others) media[4]. Immobilized lipases with enhanced thermal and mechanical stability can expand their utility to unusual pressure (P) and temperature (T) conditions. In this context, this study aims to investigate the application of high-pressure technologies using 1,1,1,2-tetrafluoroethane (R134a) on ET2.0 immobilized silica supports, specifically elucidating alterations in the secondary structure through Fourier Transform Infrared (FTIR) spectroscopy self-deconvolution and evaluating the catalytic potential in olive oil hydrolysis reactions.

2 MATERIAL & METHODS

2.1 Materials

In this work, Eversa® Transform 2.0 was used with 24.4 mg protein/mL obtained from Novozymes (Araucária, Paraná). The functionalizing agent (3-Aminopropyl) triethoxysilane (APTES) was obtained from Sigma-Aldrich Co (Missouri, USA). Silica gel (230-400 mesh) was obtained from Neon (São Paulo, Brazil). All other reagents were of analytical grade. The 1,1,1,2-tetrafluoroethane (R134a) was obtained from Dufrio.

2.2 Synthesis of Amine-silica (AmG)

The synthesis of the support was carried out following the reported procedure in the literature with few modifications[5].

2.3 Immobilization of Eversa® Transform 2.0 on Amine-glutaraldehyde-silica (AmG)

The immobilization process was carried out following the reported procedure in the literature with few modifications[5].

2.4 High pressure Apparatus and experimental procedure

The experimental setup is based on a static synthetic method using a high-pressure variable-volume view cell. The apparatus comprises a solvent reservoir (R134a), two thermostatic baths (Spencer), a syringe pump (260D, Teledyne ISCO), a stainless-steel cell with a total volume of 42 mL and a useful volume of 30 mL, equipped with a sapphire window, and an absolute pressure transducer (Smar, LD301). For each experiment, 0.5 g of AmG biocatalyst was loaded into the cell, and the system was pressurized under different conditions. The AmG was exposed to different pressure levels (50-150 bar), temperatures (30-50°C), and exposure times (2-7 hours), with a fixed depressurization rate of 30 bar/min, all within the variable-volume high-pressure visualization cell. After the exposure period, the hydrolytic activity of AmG in olive oil was measured to assess residual activity.

2.5 Determination of hydrolytic activity and Residual activity

The hydrolytic activity of lipase EVERSA Transform 2.0 (ET2.0) was determined in the hydrolysis of olive oil emulsion[6]. One international unit (IU) of hydrolytic activity was defined as the mass of enzyme required to release 1 µmol of Free Fatty Acid (FFA) per minute of reaction at pH 8.0 (100 mM buffer sodium phosphate), 37 °C and 5 min of reaction under continuous agitation (200 rpm), for evaluation of Residual activity.

The residual activity was measured through the ratio between the hydrolytic activity of the biocatalyst after being subjected to high pressure and the control hydrolytic activity (Biocatalyst under normal P and T conditions) according to equation 1.

$$Residual\ Activity = \frac{AtAp}{AtC}\ x\ 100 \quad (1)$$

Where AtAP is the activity after high pressure treatment and ActivityC is the activity of the biocatalyst without treatment.

2.6 Determination of secondary structure by FTIR

Fourier transform infrared spectroscopy (FTIR) spectra of supports and immobilized biocatalysts were recorded on a Cary 630 FTIR spectrometer (Agilent Technologies, Germany) equipped with an ATR accessory. The spectra were measured in a spectral range from 4000 to 650 cm^{-1} and at a spectral resolution of 4 cm^{-1}. For each spectrum, 32 scans were added. For secondary structure analysis, the spectra were recorded between 1700 to 1600 cm^{-1}. Evaluation of secondary structure changes included secondary derivative and deconvolution of peaks using Origin software version 8.5.

3 RESULTS & DISCUSSION

3.1 Enzymatic stability of the ET2.0 Immobilized on AmG exposed to pressurized R134a

The enzymatic stability of ET2.0 immobilized in AmG under pressurized R134a was compared with a control group (immobilized biocatalyst without treatment with pressurized fluid). The hydrolytic activity was evaluated to quantify residual activity. The results depicted in Figure 1 indicate that intermediate conditions of pressure (P) at 100 bar, temperature (T) at 40°C, and a exposition time of 4.5 hours yielded the best residual activity results, reaching approximately 250%. This suggests that under these conditions, ET2.0 immobilized was more active in the olive oil hydrolysis reaction. Conversely, the biocatalyst exposed to lower pressure (50 bar) and higher temperature (50°C) for 2 hours exhibited lower residual activity compared to the control, indicating that these conditions reduced the catalytic activity of the biocatalyst. The operational conditions employed in this study are summarized in Table 1.

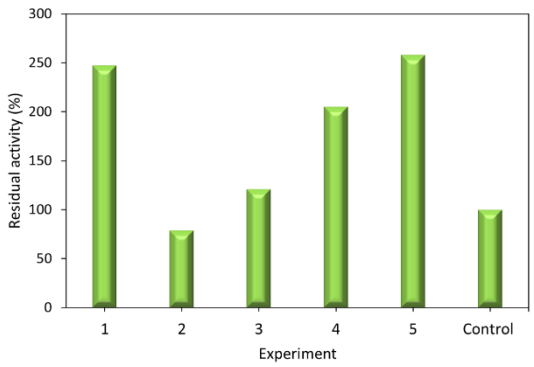

Figure 1 Enzymatic stability of the AmG biocatalyst with pressurized R134a

The results corroborate other studies in the literature where a commercially immobilized enzyme, Novozym 435, showed good results in residual activity using the solvent R134a, reaching values of 300% residual activity, and subsequently the use of this biocatalyst in an esterification reaction in pressurized medium[3].

Table 1 experimental conditions

Experiment	Conditions
1	150 bar 30°C 2h
2	50 bar 50°C 2h
3	150 bar 30°C 7 h
4	50 bar 50°C 7h
5	100 bar 40°C 4,5h
Control	1 bar 25°C

Another study reports that the solvent R134a generated a residual activity of approximately 130%; however, this result was achieved using lysozyme in its free form. The increased catalytic activity under certain operating conditions may be attributed to possible conformational changes in the enzyme's secondary structure. These changes could be linked to the enzyme's rigidity (β-Sheet) and its interaction with the lid region (α-Helix), which may expose the enzyme's active site more prominently. In the infrared spectrum, the vibrations of the amide region (A, B, I to IR) were determined within the 1600-1700 cm^{-1} band, primarily associated with the elongation of the C=O bonds of the peptide. This indicates potential changes in the secondary structure of proteins. Gaussian curves were applied to adjust this range, allowing for the identification of the contents of α-Helix (1650-1658 cm^{-1}), β-Sheet (1620-1640 cm^{-1}), β-Turn (1660-1680 cm^{-1}), and random coil (1640-1650 cm^{-1}) by area percentage, as shown in Table 2.

Table 2 Percentage composition of secondary structure of Eversa® Transform 2.0 immobilized on AmG after high pressure treatment

Experiment	β-Sheet	Random Coil	α-Helix	β-Turn
1	22.89	26.57	16.91	25.89
2	19.95	27.96	24.95	12.03
3	19.93	26.07	20.91	12.49
4	22.45	30.91	18.9	13.2
5	21.34	41.06	17.59	10.34
Control	20.00	25.29	23.21	13.78

The best results (experiments 1 and 5) presented the lowest α-Helix values, values that were much lower than the control enzyme (23.21%), values that corroborate the literature that the active site may be more exposed to the reaction medium, promoting better catalytic activity (as shown in Figure 1). In relation to β-Sheet, the best results showed an increase, where the application of P and T for a certain time caused the formation of a more rigid biocatalyst. In the literature, only one study using free lipase has the evaluation of the secondary structure after an evaluation of the enzymatic stability at high pressure, and the results obtained in this work corroborate this work, where the structure of the α-Helix had a reduction and the β- Sheet an increase for the best result obtained[2].

4 CONCLUSION

The results obtained in this study demonstrate that R134a is an effective solvent for enhancing both catalytic activity and conformational changes in ET2.0 immobilized on AmG support. Operating conditions of 100 bar and 40°C for 4.5 hours resulted in the highest residual activity among those evaluated, achieving approximately 260%. This research highlights the potential benefits of using high-pressure technologies to pre-activate immobilized biocatalysts for subsequent use in specific reactions such as esterification and hydrolysis. Additionally, other gases and biocatalysts could be explored, expanding the application of these immobilized biocatalysts to enzymatic reactions under high pressure.

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