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Short Communication
Cross-linked Enzyme Aggregates of Pig Liver Esterase Evaluated in Kinetic Resolution of Racemic Clopidogrel

[1]Yasser Gaber and [2]Mohamed Ismail

[1]Department of Microbiology, Faculty of Pharmacy, Beni-Suef University, 62514, Beni-Suef, Egypt
[2]Department of Microbiology, Faculty of Science, Helwan University, Cairo, Egypt

Abstract

Background and Objective: Immobilization of enzymes as cross-linked aggregates is one of the cheapest, simplest and effective techniques for improving their stability and reusability and even avoiding contamination of the product with the catalyst. Clopidogrel is a widely used antiplatelet drug, only *S* isomer is the biologically active enantiomer produced by resolution of the racemic compound. In the current study, cross-linked aggregates of pig liver esterase were prepared and evaluated for kinetic resolution of racemic clopidogrel. **Materials and Methods:** Cross-linked Enzyme Aggregates (CLEA) of the commercially available crude pig liver esterase cPLE were prepared using glutaraldehyde at concentrations of 12.5-125 mM as crosslinker either in presence or absence of Bovine serum albumin (BSA). cPLE-CLEA was used for kinetic resolution of racemic clopidogrel and compared to the performance of soluble cPLE. Light microscopy and scanning electron microscopy SEM were used to examine cPLE-CLEA. **Results:** Soluble cPLE showed ability to resolve racemic clopidogrel at enantioselectivity (E) of 9.2. The resolution of clopidogrel was found to be optimal at 30°C. The cPLE-CLEA preparations showed reduced enzymatic activity. The kinetic resolution experiments showed also lower E values (E = 1.3-4.5) compared to soluble cPLE. Microscopical examination of cPLE-CLEA showed wide size variation and SEM revealed the shape of cPLE-CLEA before and after use in the kinetic resolution experiments. **Conclusion:** Crude PLE was able to resolve racemic clopidogrel, the effects of different temperatures were studied and the highest E value recorded was 9.2 at 30°C. Increasing concentrations of glutaraldehyde as a cross-linker adversely affected PLE activity. The cPLE-CLEA showed lower enantioselectivity compared to the free cPLE.

Key words: CLEA, esterase, enantioselectivity, glutaraldehyde, clopidogrel, kinetic resolution

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Corresponding Author: Yasser Gaber, Department of Microbiology, Faculty of Pharmacy, Beni-Suef University, 62514, Beni-Suef, Egypt
Tel: 00201200136133/002-0822162133 Fax: 002-0822162133

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Clopidogrel is one of the most prescribed antiplatelet medications[1]. Only the S-isomer is the active form of this compound, while the R-isomer has no antiplatelet activity[2]. A route commonly used for its synthesis is the preparation of racemic clopidogrel followed by diastereomeric resolution using stoichiometric amounts of camphor sulfonic acid. Recently, the possibility of enzyme-catalysed kinetic resolution of racemic clopidogrel was reported[3,4]. An enzyme based process would reduce the volume of chemicals, solvents and energy consumed in the conventional process, however, for it to be applicable requires optimization of the biocatalyst with respect to several parameters including activity, stability, enantioselectivity and cost.

Immobilization of enzymes is an important mean to facilitate their application by improving their stability and reusability and hence the economics of the processes besides also minimising the risk of product contamination and potential allerginicity. Different approaches are available for immobilization of enzymes including adsorption or covalent linking to a support. Immobilization as cross-linked aggregates provides a simple and effective approach that has attracted attention in enzyme technology[5]. Cross-linked enzyme aggregates (CLEAs) are preparations where the enzyme is precipitated and then cross-linked using a bi-functional agent such as glutaraldehyde. CLEAs are easy to prepare and usually improve enzyme performance as compared to soluble enzymes[5]. CLEAs usually show altered properties in terms of selectivity and activity[6]. Pig liver esterase is a highly versatile and one of the most used enzyme for chemical transformations at large scale. Crude PLE (cPLE) catalyses enantioselective hydrolysis of many classes of compounds[7]. Crude PLE has been described in the literature as a mixture of seven isozymes with differences in enantioselectivities[8-12].

Crude PLE was reported earlier to be among few euzymes showing activity on recamic clopidogrel[4]. In the current report, the use of cPLE in free- and immobilized form as CLEA was evaluated as a biocatalyst for kinetic resolution of racemic clopidogrel.

MATERIALS AND METHODS

General: All the experiments were performed at Lund University, Sweden during the period 2011-2014. Racemic clopidogrel ester (free base) as a light brown viscous oil (purity > 99%) was kindly provided by Sun Pharma, India. Crude pig liver esterase (16 U mg^{-1}) and glutaraldehyde (25 wt% in H_2O) were obtained from Sigma Aldrich. DMSO, analytical grade, was purchased from Prolabo (Paris, France). Methanol and acetonitrile and HPLC grade were purchased from Merck, Darmstadt, Germany.

CLEA preparation: Two batches of CLEAs were prepared; the first involved crosslinking of crude PLE with Bovine serum albumin (BSA) at a molar ratio 1:1 using solutions of 1 mg mL^{-1} PLE and 0.4 mg mL^{-1} BSA, while the second batch was based on cPLE only. The molecular weight of crude PLE was assumed to be 168 kDa and that of BSA 69 kDa. The formation of enzyme aggregates was induced by a high concentration of ammonium sulphate solution (40% w/v). Glutaraldehyde was used at six different concentrations (12.5, 25, 60, 80, 100 and 125 mM) for crosslinking, The suspensions were incubated with shaking in a thermomixer (HLC, Germany) at 4°C for 3 h, after which the preparations were centrifuged at 10000 rpm for 2 min and washed twice with phosphate buffer pH 7.4. Finally the cPLE-CLEAs were kept suspended in 200 µL phosphate buffer at 4°C untill use.

Assay of esterase activity: Ethyl butyrate assay was performed to determine the activity of the soluble cPLE and cPLE-CLEA preparations. The assay was done according to the Sigma Aldrich protocol using ABU-901 Auto Burette (Radiometer, Denmark). Soluble cPLE or cPLE-CLEA preparations (5-15 mg) were put in the titration vessel containing 25 mL of 10 mM borate buffer equilibrated at 25°C, followed by incremental addition of ethyl butyrate solution.

Kinetic resolution of racemic clopidogrel: Racemic clopidogrel dissolved in DMSO was added at a concentration of 1-50 mM phosphate buffer containing 1 U mL^{-1} of the different cPLE-CLEA preparations based on the ethyl butyrate activity assay, the DMSO concentration was 10% v/v and the final reaction volume was 0.3 mL. Reactions were stopped after 9 h by adding 0.6 mL of methanol, filtered and analyzed by HPLC.

HPLC analysis: Analysis of the reaction mixture was performed according to Nikolic *et al.*[13], using Jasco HPLC system, Chiradex column (5 µ, 4×250 mm, Merck, Darmstadt, Germany) thermostatted at 17°C and a mobile phase comprising acetonitrile, methanol and 0.01 M potassium dihydrogen phosphate solution (15:5:80 v/v/v), at a flow rate of 1.0 mL min^{-1}. Injection volume of the sample was 20 µL and the eluate was monitored at 220 nm on a UV detector (Jasco,

model UV970/975). Substrate conversion and enantiomeric excess (% excess of one pure enantiomer over the other) was calculated according to Chen *et al.*[14].

Scanning electron microscopy: The CLEA preparations were sputter-coated with gold/palladium (40/60) and inspected using a scanning electron microscope JEOL JSM-5000LV (JEOL Ltd., Akishima, Tokyo, Japan).

RESULTS AND DISCUSSION

The current study reports for the first time preparation of cross-linked enzyme aggregates of crude pig liver esterase. The effect on cPLE activity and enantioselectivity was assessed based on evaluation of CLEAs ability to resolve racemic clopidogrel compound in comparison to free cPLE. Based on previous studies, cPLE selectively hydrolyse racemic clopidogrel with certain enantioselectivity in comparison to other esterases[4]. Prior to making the CLEAs, the influence of temperature on the reaction using soluble cPLE was investigated. As shown in Table 1, while the degree- and rate of conversion increased with temperature, the highest enantiomeric excess and E value (9.2) was highest at 30°C. The increase in reaction temperature resulted in decrease in the observed enantioselectivity (E = 3.7 and 2.7 at 40 and 50°C, respectively). This observation is in agreement with the study carried out by Yousefi *et al.*[15]. The study has shown the effect of different temperatures on the enantioselective resolution of racemic ibuprofen using different lipases. On the other hand, Sakai *et al.*[16] have shown that the enantioselectivity of lipase-catalysed transesterification reaction performed in an organic solvent to be highest (E = 99) at very low temperature (- 40°C).

For preparation of CLEAs, the cPLE was precipitated using high concentration of ammonium sulphate and then cross-linked with different concentrations (12.5-125 mM) of glutaraldehyde. The preparations made with 12.5 mM glutaraldehyde showed the highest esterase activity (0.13 U mg^{-1} using ethyl butyrate assay) compared to other preparations cross-linked with higher concentrations of glutaraldehyde (Fig. 1). In a previous study, *Pseudomonas putida* nitrilase has been prepared as CLEAs and was tested for enantioselective nitrile hydrolysis reaction. The CLEA preparations retained 70% enzymatic activity upon using 125 mM glutaraldehyde as a crosslinker[17]. Glutaraldehyde is a bi-functional crosslinker reacting with the free amino groups on the amino acids (e.g. lysine) in the proteins and the optimum concentration needed for making CLEAs has to be optimized on a case-to-case basis[18]. For example,

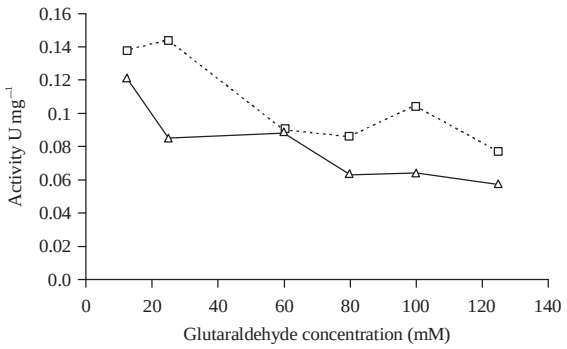

Fig. 1: Effect of different concentrations of glutaraldehyde on the activity of CLEA preparations made with crude pig liver esterase alone (dashed line) and in presence of BSA (solid line), respectively

Table 1: Effect of temperature on the activity and enantioselectivity of crude pig liver esterase on the hydrolysis of racemic clopidogrel

Temperature (°C)	Time (h)	Conversion (%)	ee (%)	E
5	12	60	79	7.5
10	6	56	74	8.0
30	3	67	95	9.2
40	1	73	77	3.7
50	1	94	94	2.7

ee%: Enantiomeric excess of the racemic clopidogrel mixture after enzymatic hydrolysis, E: Enantioselectivity of the crude PLE as calculated by Chen *et al.*[14]

Perzone *et al.*[19] have shown that CLEA-cellulase prepared with glutaraldehyde at concentrations of 30 and 100 mM retained high activity when polyethylene glycol was used as a precipitant. On the other hand, CLEA-cellulase prepared with glutaraldehyde at a concentration of 5 mM retained high activity when ammonium sulphate was used as a precipitant[19].

Table 2 shows the results of the kinetic resolution of racemic clopidogrel catalysed by with different cPLE-CLEA preparations at 30°C (entries 3-26) in comparison with soluble cPLE (entries 1-2). It was observed that cPLE-CLEA entries showed variations in the values obtained of conversions for same preparations, e.g., the entries 7 and 8 showed conversions of 36 and 81 %, respectively. This is related to the wide variation in the CLEA particles as shown by the microscopic images in Fig. 2. The variation has also been observed in the ee % and E values recorded for the different entries (Table 2). It has been reported in literature that CLEAs could form clusters that lead to variation in the particle sizes[20]. In a recent study, CLEA preparations in compartitions to other different immoiblization techniques have been evaluated on the effect of the enantioselectivity exihibited by *Candida rugosa* lipase. The study attributed the differences in the observed enantioselectivity to the conformational changes in the enzyme active site and the effect of the reaction medium

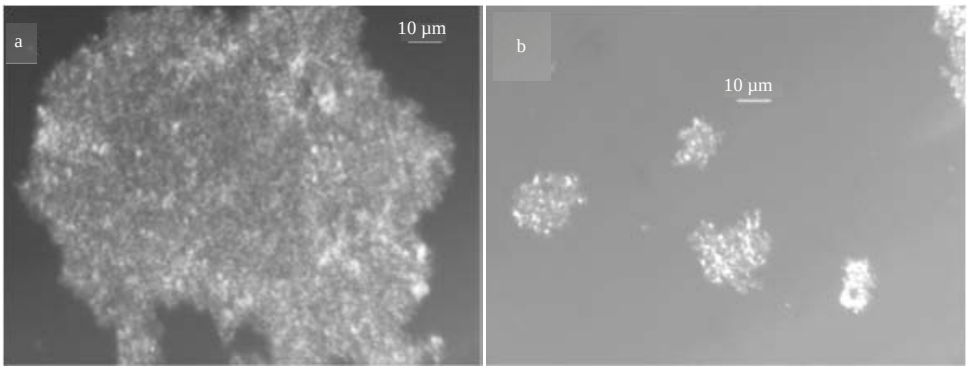

Fig. 2(a-b): Size variation of the cPLE-CLEA particles as seen in two different microscopical fields a and b (the bar in the images is 10 µm in length)

Table 2: Activity and selectivity of different preparations of cPLE-CLEAs against racemic clopidogrel

		Kinetic resolution of racemic clopidogrel		
Entry	Glutaraldehyde concentration mM	Conversion[a] (%)	ee[b] (%)	E[c]
Crude PLE				
1	None	49	65	10.4
2		54	75	9.7
CLEA of crude PLE+BSA				
3	12.5	59	32	2.0
4		56	56	4.5
5	25	36	30	4.3
6		69	46	2.2
7	60	36	15	2.0
8		81	19	1.3
9	80	29	20	3.5
10		62	33	2.0
11	100	51	21	1.8
12		28	19	3.4
13	125	33	18	2.5
14		56	27	1.9
CLEA of crude PLE				
15	12.5	47	39	3.7
16		84	75	2.5
17	25	52	37	2.9
18		66	39	2.1
19	60	54	31	2.3
20		37	22	2.6
21	80	40	17	2.0
22		30	16	2.5
23	100	44	21	2.1
24		43	22	2.2
25	125	49	20	1.8
26		50	22	1.9

[a]The kinetic resolution reactions using CLEA preparations were carried out at 30°C, CLEA preparations was added to the reaction mixture to give final activity of 1 U mL^{-1} of cPLE based on ethyl butyrate assay, the reactions were stopped after 9 h using 1 mL of methanol, [b]ee%: Enantiomeric excess of the racemic clopidogrel mixture after enzymatic hydrolysis, [c]E: Enantioselectivity of the crude PLE as calculated by Chen *et al.*[14]

conditions i.e., co-solvent and temprature[21]. In another biocatalytic application, Chun-Yang *et al.* have prepared CLEAs of epoxide hydrolase in order to selectively resolve styrene oxides. A range of glutaraldehyde concentrations (10-25 mM) and different precipitants were used in the formulation of the CLEAs[22].

Imaging the CLEA particles with scanning electron microscopy showed the difference of the preparations before and after use in the chemical reaction (Fig. 3). CLEA preparations appeared ball shaped with rough surface before use in the chemical reaction, while the surface became smoother after use, probably due to being coated by the

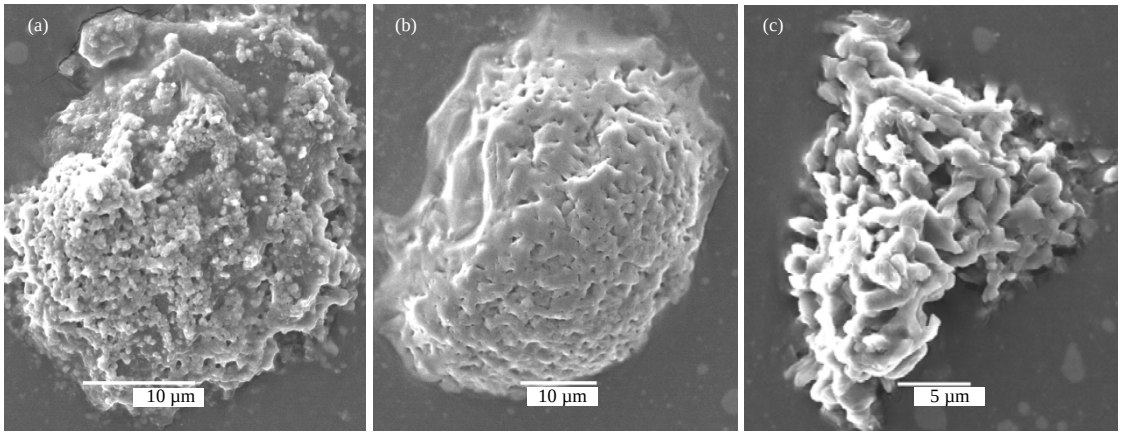

Fig. 3(a-c): Scanning electron microscopy (SEM) images of CLEA preparations before use (a) and after use (b) in the kinetic resolution reaction. Zoom-in to the shape of CLEAs is illustrated in (c)

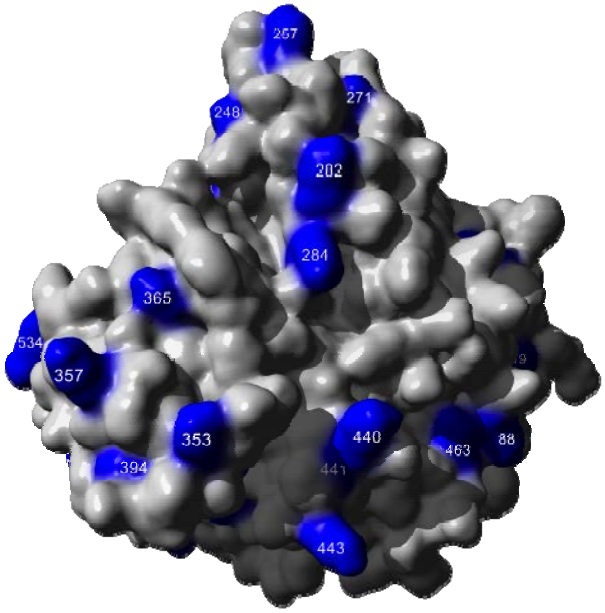

Fig. 4: Screenshot of PLE-1 model in surface representation shows the distribution of lysine residues present on the enzyme surface

reaction mixture containing the substrate. This may also contribute towards blocking the active site and reduction in CLEA activity. Such an effect might be overcome by introducing a suitable washing step to allow removal of the adhered material and open paths to the entrance of the substrate to the enzyme active sites.

A previous study has confirmed that although PLE-1 is the most abundant isozyme present in the crude preparation, it is not responsible for the enantioselectivity exhibited by cPLE towards clopidogrel[4]. Structural and sequence comparison of the different PLE isozymes suggested that PLE-3 or PLE-5 might be the ones that have the enantioselectivity towards racemic clopidogrel. The crosslinking reaction of cPLE molecules is expected to change the distribution of activities of the functional isozymes within the crude PLE. The decrease in enantioselectivity towards racemic clopidogrel may be due to possible inactivation of the isozyme responsible for the enantioselectivity exhibited by the crude PLE. Adding BSA to crude PLE prior to crosslinking did not notably improve the PLE enantioselectivity of the CLEA preparations. BSA has earlier been reported in the formulation of CLEAs since it can protect the enzyme active sites and act as a diluent in the CLEA preparation[23]. The BSA also acts as a spacer between enzyme molecules and increases the intermolecular spaces between cross-linked molecules. Guauque Torres *et al.* have also used BSA in a different approach in order to improve the enzymatic activity retained and reduce the mass transfer limitation encountered in ordinary BSA-CLEA preparations. The approach is named layered methodology, where a core of cross-linked BSA was prepared first followed by crosslinking to the desired enzyme. The study showed the high efficiency of the layered *Candida antarctica* lipase B CLEAs in the fatty acid esterification reactions[24].

The 3D structures for PLE isoenzymes have not yet been determined. PLE-1 is the most abundant isozyme in the cPLE according to Hummel *et al.*[25]. YASARA Structure software was used for building a homology model of PLE-1 and for further structural analysis. According to the topology analysis and amino acid residues distribution on the surface of PLE-1, 36 lysine residues are present in PLE-1 sequence and almost the same number is found also in the other PLE isozymes. These lysine residues are mostly located on the protein surface (Fig. 4), which would provide good sites for interaction with glutaraldehyde and would likely result in high degree of

crosslinking, reducing the access of the substrate to the active site and consequent decrease in activity.

CONCLUSION

Although, there have been many reports on the robustness and stability of CLEAs, there is not much information on the effect on enzyme enantioselectivity. The current study has shown the enantioselectivity of crude PLE to be lowered when prepared in the form of cross-linked enzyme aggregate. Advancement in biocatalysis requires improvement in enzymatic properties, especially stability and reusability. CLEAs preparations with improved properties have been achieved in other studies for gram scale preparation of products and even when using more complex systems with several enzymes in what are known as Combi-CLEAs. In the present study, although the E-value was lowered, the cPLE-CLEAs preparation could be reused several times and catalyse the kinetic resolution reactions which is considered beneficial for industrial applications. Further benefit would be a less likelihood of PLE protein being left in the final product. This is important within the context of pharmaceutical industry that requires strict control of contamination in the final drug formulation.

SIGNIFICANCE STATEMENTS

Enzymes are of great value in preparation of chiral chemicals such as pharmaceuticals. Hence, the demand for selective and robust enzyme preparations is high especially for chemical synthesis. The current report investigated the efficacy of enzyme immobilization as cross-linked aggregates on the enantioselectivity of crude pig liver esterase. The enantioselectivity was tested on racemic clopidogrelone of the most used antithrombotic agents worldwide.

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