

# Isolation of *Candida rugosa* lipase isoforms

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## Abstract

The yeast *Candida rugosa* is a convenient source of lipases for science and industry. Crude preparation of *Candida rugosa* lipase (CRL) consists of several extracellular lipases. Isoenzyme profile depends on the culture or fermentation conditions. All isoforms are coded by the lip pseudogene family; they are monomers of 534 amino acids and molecular weight of about 60 kDa. They share the same catalytic mechanism and interfacial mode of activation. Isoenzymes differ in isoelectric points, post-translational modifications, substrate specificity and hydrophobicity. The presence of different lipase isoforms and other substances (*i.e.*, inhibitors) in crude preparation leads to lack of their productivity in biocatalytic reactions. Purification of specific isoform improves its overall performance and stability. This paper provides an overview of different methods for purification of CRL isoenzymes up to date, their advantages and disadvantages.

**Keywords:** Lipases, *Candida rugosa*, isoforms, purification.

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Lipases (E.C.3.1.1.3, glycerol ester hydrolase) catalyze the hydrolysis of an ester bond between a fatty acid and glycerol. They are among the most important biocatalysts in modern science and industry. Many plant, animal and microbial cells produce lipases. Microbial lipases (fungal, bacterial or yeast) have more importance over animal and plant lipases, because they can be easily grown on inexpensive agricultural waste materials: sugarcane, plant or animal fats and oils, etc. [1–6].

Yeast *Candida rugosa* (formerly known as *Candida cylindracea*) is a non-sporogenic, pseudofilamentous, unicellular yeast and imperfect hemiascomycete. It grows aerobically in nutritive media with different carbon and nitrogen source. *C. rugosa* is non-pathogenic microorganism, and its lipases do not show genotoxic or cancerogenic effects on human health. Because of that, the species is generally regarded as safe (it has GRAS status), and classified as biological safety level 1 [7–9].

CRL are decoded by lip pseudogene family. Each isoenzyme is a product of the expression of an individual gene, and each of the lip genes is transcriptionally active [7]. *C. rugosa* can produce 5–7 lipase isoforms, depending on the culture or fermentation conditions. All of them are glycosylated monomers of 534 amino acids and approximate molecular weight of 60 kDa and have the same catalytic triad (Ser, His, Glu or Asp). Iso-

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enzymes differ in their isoelectric points, carbohydrate content, substrate specificity and hydrophobicity.

*C. rugosa* lipases are very important biocatalytic tool in different fields of industry (food processing, cosmetic and pharmaceutical preparations, biodiesel production and detergent industry) and science (environmental sciences, analytical chemistry and medicine) [10–17].

## EXPERIMENTAL

Crude preparation of CRL is a mixture of several different lipases and esterases. Activity of one of these enzymes affects the others in the preparation (*i.e.*, in a competitive way), so it is necessary to purify the individual isoform in order to improve its catalytic properties [18].

### Multi-step approach

Pernas *et al.* purified an esterase of about 43 kDa and reported separation of lipase isoforms 2 and 3 from crude CRL preparation [19]. The crude sample was prepared in two ways: in the first preparation, the sample was simply dissolved in buffer and centrifuged. That supernatant was subjected to hydrophobic interaction chromatography (HIC) on phenyl-sepharose, followed by anion-exchange chromatography on DEAE-sephacel and gel-filtration on sephacryl S-200. The second preparation was slightly different, since the crude sample was dissolved in a buffer that contains ammonium sulphate. After stirring and centrifugation, obtained supernatant was subjected to HIC on phenyl-sepharose HR5/5, followed by anion-exchange chroma-

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tography on Source 15Q HR5/5 and gel-filtration on sephacryl S-200.

Sailas and Pandey described purification method for three distinct fractions of lipases from crude fermentation material, designated as lipase A, B and C [20]. The purification scheme consists of: ammonium-sulphate precipitation, dialysis, ultrafiltration and gel-filtration on sephadex G-200.

Combining chromatography on agarose-mercurial column and desalting step on sephadex G-25, Tai *et al.* reported separation of two main lipase fractions from crude sample [21].

Combination of anion-exchange chromatography on DEAE-Sepharose CL-6B and “hydrophobic interfacial affinity chromatography” resulted in fine resolution of CRL isoenzymes, according to Xin *et al.* [22]. The anion-exchange step serves for crude separation of two main lipase fractions: CRL A and CRL B. HIC on nucleosil C4 column allowed fine separation of different isoforms, present within the main fractions. Accordingly, both CRL A and CRL B were further resolved to 4 isoenzymes, which slightly differ in their hydrophobicity pattern and hence cannot be separated by conventional HIC methods (*i.e.*, HIC on phenyl-sepharose). In isoelectric focusing, however, CRL A fraction only gave a single band of approximate  $pI = 5.6$  and CRL B fraction also gave a single band of an approximate  $pI = 4.2$ .

Lipase 3 isoenzyme is reported to act on cholesteryl-esters. In order to investigate its activity on different plant steryl-esters it was necessary to obtain it from crude preparation. Tenkanen *et al.* describe a method for purification of this lipase isoform, based on combination of classical pre-purification procedure, HIC on phenyl-sepharose FF and ion-exchange chromatography on DEAE-Sepharose [23].

Blends of alginate and gelatin, additionally functionalized with succinic anhydride, can be used for separation of lipases, according to procedure described by Fadnavis *et al.* [24].

### One-step approach

In recent years there has been a growing interest for developing new, more economic one-step purification strategies. In the further text, some of them will be presented.

Lv and associates have reported a one-step method for successful resolution of *Candida sp.* lipase into four distinct fractions [26]. In this approach, they used ion-exchange chromatography on polyethyleneimine monoliths.

Solanki and Gupta [26] have reported simultaneous immobilization and purification of CRL, for the use in tranesterification. Lipase was adsorbed on a  $Fe_3O_4$  nanoparticles and coated with polyethyleneimine (PEI). It is believed that purification is achieved because of selective binding property of PEI.

### RESULTS AND DISCUSSION

The general advantage of a multi-step purification approach is that there are well established purification protocols, which are freely available. Also, a great advantage is the use of classic purification techniques, such as ion-exchange chromatography, HIC, gel-filtration, precipitations, etc.

On the other hand, the main disadvantage of such purification methods is the low yield of purified isoenzymes. Table 1 summarizes some of the previously mentioned methods with purification yields. According to data from the table, the highest yield is achieved after purification strategy described in literature [20]. However, in terms of industrial use, the yields are too low, so the described methods are not profitable for large-scale production. Apart from the low yield, the duration of purification process is also a problem, because at least 3 steps are needed for purification of an isoenzyme of interest.

One-step purification methods are convenient for modern approach in isolation of lipases from their sources. They are usually easy to perform, fast and

Table 1. Purification yields of different multi-step purification strategies

Purification steps	Purified isoenzyme	Yield, %	Reference
Precipitation, sodium-cholate treatment, ethanol/ether, anion-exchange	Lip 2	4.3	19
	Lip 3	4.9	
Ammonium-sulphate precipitation, dialysis, ultrafiltration, gel-filtration	Lip A	11.62	20
	Lip B	1.15	
	Lip C	3.64	
Chromatography on agarose-mercurial column	CRL I	Not defined	21
	CRL II		
	CRL III		
Ion-exchange chromatography, hydrophobic interfacial affinity chromatography	CRL A	Not defined	22
	CRL B		
Pre-purification, HIC, ion-exchange	Lip 3	7	23

inexpensive. In the term of yield, the represented one-step strategies are compared in Table 2.

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Table 2. Purification yields of different one-step purification strategies

Purification steps	Purified Isoenzyme	Yield, %	Reference
Ion-exchange chromatography on polyethyleneimine monolith	Lipase A	3.50	25
	Lipase B	19.8	
	Lipase C	16.6	
	Lipase D	12.3	
Simultaneous immobilization and purification	Not defined	Not defined	26

## CONCLUSION

Within the last decade, interest for microbial lipases, especially those produced by *C. rugosa*, has increased and will probably continue in that trend even more so in the future. Because of their numerous applications, especially in different fields of industry, it is necessary to find a way to purify these valuable biocatalysts from the crude preparations, which are most commonly used as starting material. Purification increases biocatalytic performances of an enzyme, since it is released from impurities, which can have inhibitory effects on its activity. Having in mind different approaches, described in this paper, it is possible to conclude what their advantages or disadvantages are.

Different multi-step methods have been described and developed, but their major disadvantage is a great loss in the yield during several purification steps. This disables the use of such approaches in large-scale purification, since it is expensive, time-consuming and gives poor yield of the enzyme of interest.

One-step approaches have also been developed. They are less time consuming, but not all of them are cost-effective, since some of them use expensive affinity ligands, or matrices, which cannot be used in large-scale production. The purification yield of such approaches can be improved by developing new, more selective purification procedures.

## Nomenclature

CRL – *Candida rugosa* lipase

DEAE – Diethylaminoethyl group

EC – Enzyme Commission

FF – Fast flow

HIC – Hydrophobic interaction chromatography

HR – High resolution

Lip – Lipase

PEI - Polyethyleneimine

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## IZVOD

### IZOLOVANJE IZOFORMI LIPAZE IZ *Candida rugosa*

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(Stručni rad)

Lipaze (hidrolaze estara glicerola, E.C.3.1.3.3) su važna grupa enzima, široko rasprostranjenih u prirodi. Mogu se izolovati iz materijala biljnog, životinjskog ili mikrobnog porekla. Zahvaljujući svojim karakteristikama, pobuđuju sve više pažnje kao efikasni biokatalizatori u različitim sintetičkim i hidrolitičkim procesima. Među lipazama, poreklom iz mikroorganizama, posebno su značajne one koje proizvodi kvasac *Candida rugosa*. Komercijalni preparat lipaza iz *C. rugosa* može sadržati 5–7 izoformi ekstracelularnih lipaza. Sve te izoforme kodirane su od strane *lip* familije pseudogena, a na njihovu ekspresiju utiču uslovi u kojima se mikroorganizam gaji (sastav hranljive podloge je najvažniji). Ekstracelularne lipaze, koje proizvodi *C. rugosa* su monomerni glikoproteini, molekulske mase od oko 60 kDa, sa 534 aminokiseline. Za sve izoforme je karakterističan isti složeni mehanizam aktivacije na granici faza i mehanizam katalize, kakav se sreće i kod serin-proteaza. Izoenzimi se međusobno razlikuju po post-translacionim modifikacijama (udelu ugljohidratne komponente), supstratnoj specifičnosti, izoelektričnim tačkama i hidrofobnosti. Prisustvo više izoformi lipaza u komercijalnom preparatu utiče na njihovu produktivnost u reakcijama koje katalizuju. Takvi preparati često sadrže i druge supstance koje mogu uticati na aktivnost enzima (na primer inhibitori). Razdvajanjem pojedinačnih izoformi iz komercijalnog preparata poboljšavaju se njihova enantioselektivnost, specifična aktivnost i stabilnost enzima, što je od izuzetnog značaja za njihovu dalju primenu. U ovom radu su predstavljeni različiti pristupi u razdvajanju pojedinačnih izoformi vanćelijskih lipaza iz komercijalnog preparata *C. rugosa*, njihove prednosti i nedostaci.

**Ključne reči:** Lipaze • *Candida rugosa* • Izoforme • Prečišćavanje