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Monoclonal antibodies against *Candida rugosa* lipase

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Abstract

We generated monoclonal antibodies (MoAbs) BF11 and VNH9 against *Candida rugosa* lipase (CRL), one of the most widely used lipolytic enzymes. The antibodies, both of the IgG_1 isotype recognize specie specific and distinct antigenic determinants shared by different CRL isoforms present both in the native and in the denaturated form of the enzyme. The CRL enzyme maintained its enzymatic activity even when immunocomplexed with MoAb BF11 and VNH9. These novel reagents are of interest for their possible application as immobilization and purification systems, and during fermentation processes.

Keywords: *Candida rugosa* lipase; Microbial lipase; IgG_1 isotype

1. Introduction

Microbial lipases of different sources find interesting applications in organic synthesis, because they couple a broad substrate-specificity to a high stereoselectivity and regioselectivity, are easily available, and do not require cofactors [1,2].

Candida rugosa lipase (CRL) is one of the most used enzymes in bioindustry since it is safe and non sporogenic [3–5]. The lipolytic enzyme is aspecific in the hydrolysis of triglycerides, is able to synthesise primary and secondary esters and accepts as substrates esters at different acylic chain length.

Monoclonal antibodies (MoAbs) that selectively recognize molecules can contribute to the understanding of the lipase structural characteristics and the kinetics of enzyme activation as well as being of wide interest for their possible biotechnological applications [6,7]. The generation of MoAbs specific for CRL lipases have not yet being described.

2. Results and discussion

MoAbs were generated by fusing splenocytes from Balb/c immunized mice with murine myeloma NS-2 cells. Hybridoma cultures were screened in ELISA for antibody production specific for CRL used as immunogen. Fig. 1 shows the reactivity of two MoAbs selected, both of the IgG_1 isotype in ELISA and Western Blotting experiments. MoAb BF11 and VNH9 reacted strongly with all the CRL preparations containing different lipase isoforms (Lip1, 2 and 3) purified in different laboratories (panel A). The reactivity was maintained also in denaturing conditions (panel B) as shown by the reacting bands that migrate in SDS–PAGE at the expected molecular weight range of 60 kDa.

The three isoforms (Lip1, 2 and 3) display a high sequence homology up to 84% where the differences are scattered in a few unique amino acids along the entire sequence [8]. Recombinant Lip 1 expressed in *Pichia pastoris* was also tested in ELISA and results show that MoAb BF11 reactivity was abolished whereas the binding of MoAb VNH9 was strongly reduced. Since the *Lip1* gene is entirely transcribed in the recombinant form and the enzyme is active as in the native form, most probably the different reactivities are to be ascribed to the glycosylation occurring in *Pichia pastoris* yeast. The *N*-glycosylation which is shared among all isoforms and is therefore possibly involved, is at position 351.

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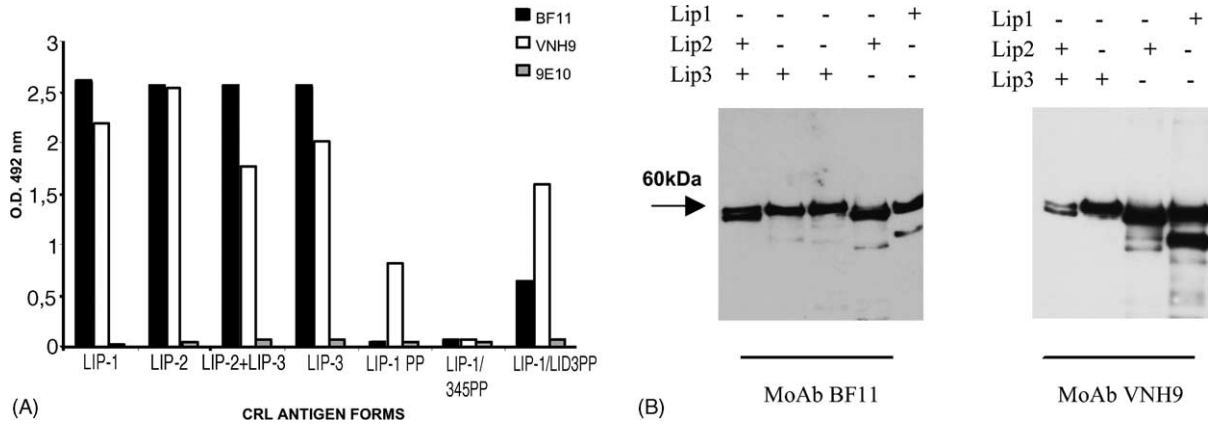

Fig. 1. Reactivity of MoAbs BF11 and VNH9 against native and recombinant CRL. (A): MoAbs BF11 and VNH9 specificity assayed by ELISA using Lip1, 2 and 3 and recombinant CRL produced in *Pichia pastoris* yeast. MoAb 9E10 was used as negative isotype matched control and binding was evaluated in excess of antigen. (B): Western blotting analysis of different isoforms of CRL antigen performed in reducing conditions.

A mutated form of the recombinant Lip-1 protein that has an aminoacid substitution in position 345 (Phe345 → Val) resulted in the abrogation of the VNH9 reactivity thus indicating that this position was important in determining the VNH9 epitope. A third recombinant form of Lip-1 was also tested, a chimera resulting from the combination of Lip-1 with the lid from the Lip-3 isoform. Results reported in Fig. 1 panel A indicate that in this case reactivity is restored partially for MoAb BF11 and almost quite completely for MoAb VNH9. This recombinant lipase has essentially six aminoacid differences (the differences between the two lids of Lip1 and 3). One of them Tyr 69 present in Lip1 is substituted in the Lip3 isoform with Phe 69. It is postulated that in Lip1 this tyrosine is involved in the interaction with the sugar moiety Asn 351 to stabilize the lipase lid opening [9]. In the recombinant chimeric lipase the substitution of Tyr 69 with Phe would affect this interaction. Once again results appear to suggest that position 351 with its carbohydrate structures can be involved either directly or by configuration properties with the epitope recognized by both antibodies. In addition position 345 appears to significantly contribute to the antigenic determinant of MoAb VNH9.

In order to define the expression of the recognised antigenic determinant also on lipases from other species, ELISA was conducted and results are shown in Table 1.

Reactivity of MoAb BF11 and VNH9 appears to be restricted to the lipase from the *Candida rugosa* specie. All lipases purified from other yeasts (*Candida antartica*, isoform A and B; *Rhizomucor miehei*, *Rhizomucor oryzae*), from bacteria (*Pseudomonas cepacea*), from plants (Wheat germ) or from mammalian (Pancreatic porcine) as well as other enzymes such as the plant-derived pectinase do not react with the MoAb.

All of these lipase belong to the super family of alpha/beta hydrolase fold proteins that have a His–Acid–Ser triad where examples exist for both Asp or Glu as the acid. They share the ability to perform interfacial activation [10] and in many cases the presence of an opening lid. The sequence homology is very low even among fungal lipase family so the high specificity observed for MoAbs BF11 and VNH9 it is not surprising.

Table 1
ELISA reactivity of MoAbs BF11 and VNH9 with enzymes from different sources (O.D. 492 nm)

	BF11	VN2H9
Candida rugosa lipase (immunogen)	2.242	2.206
Candida antartica lipase	0.102	0.105
Candida antartica A Isoform lipase	0.118	0.114
Candida antartica B Isoform lipase	0.102	0.150
Pseudomonas cepacea lipase	0.141	0.06
Rhyzomucor miehei lipase	0.294	0.120
Rhyzopus Oryzae lipase	0.196	0.058
Pectinasis	0.157	0.074
Wheat Germ lipase	0.31	0.068
Pancreatic porcine lipoase	0.185	0.061

A possible functional effect of MoAbs binding on the lipolytic activity was studied evaluating enzyme activity in solution and on PAGE before and after the formation of the antigen–antibody complex. CRL when complexed to purified MoAb BF11 at different enzyme/antibody ratio maintains its lipolytic activity as assayed in hydrolysis reaction of tributyrrin. Similar results were obtained employing MoAb VNH9. In Table 2 the residual activity obtained for both MoAbs in the same condition and enzyme ratio are summarized. These results indicate that the lypolitic activity of CRL is not affected by MoAb interaction.

The native PAGE technique was applied to simultaneously detect MoAb-enzyme complex formation and the esterasic

Table 2
Residual activity in hydrolysis reaction after MoAbs binding

Enzyme	Residual activity (%)
CRL-UAB	100
CRL-UAB + MoAb BF11	99
CRL-UAB + MoAb VNH9	92

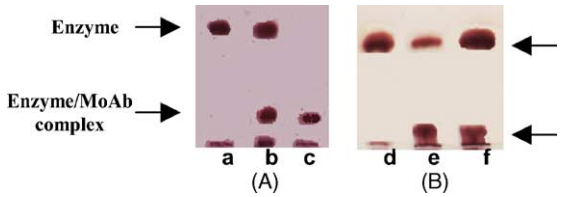

Fig. 2. PAGE in native condition of CRL and CRL complexed with MoAb BF11 stained for esterasic activity. (A): Lane a: Lip1; lane b: Lip1 + MoAb BF11 1:1 ratio; lane c: Lip1 + MoAb BF11 1:2 ratio. (B): Lane d: Lip2; lane e: Lip2 + MoAb BF11 1:2 ratio; lane f: Lip2 + MoAb BF11 1:1 ratio.

activity on a solid phase. Samples of MoAb-enzyme complex at different ratio were incubated at 4 °C for 2 h and then loaded on polyacrylamide gels. The electrophoresis was run in native conditions and gels were stained for esterasic activity. It is interesting to observe that the addition of the MoAb results in the appearance of a new spot at high molecular weight corresponding to the immunocomplex (Fig. 2: panel A, lane b; panel B, lane f). Moreover samples at different enzyme-MoAb ratio (Fig. 2: panel A, lane c; panel B, lane e), show increase of intensity of the spot corresponding to the enzyme-MoAb complex while the low MW spot corresponding to the free enzyme progressively disappears. Similar results were obtained with MoAb VNH9. These results indicate that as for lypolitic activity esterasic activity is unmodified even when the immunocomplex with both MoAbs BF11 and VNH9 is formed. This is the first time that the PAGE method is applied to visualize capture/immobilization and activation of a lipase simultaneously. We are now generating novel MoAbs using this type of assay to specifically screen and select MoAbs that can inhibit or modulate enzyme activation.

3. Experimental

Four week old Balb/c female mice were immunized twice intraperitoneally (once with 40 μg in Complete Freund Adjuvant and once with 40 μg in Incomplete Freund Adjuvant) and once intravenously (20 μg in phosphate buffer saline (PBS)) with a semipurified preparation of CRL [12] 3 days prior fusion. Spleen cells were fused with NS-2 mouse myeloma cells.

ELISA was performed absorbing antigens in excess quantity to 96 well plates in carbonate buffer pH 9.2 at 37 °C overnight (o/n). After blocking with 5% BSA–PBS, plates were incubated with the culture supernatant for 2 h at 37 °C. Antibody binding was then revealed by incubating the plates with goat anti mouse IgG (H + L) affinity purified peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, USA) for 1 h at 37 °C. Optical density was measured at 492 nm wavelength. An unrelated isotype matched MoAb 9E10 against *c-myc* antigen was used as negative control.

Lipolytic activity of CRL was evaluated by alkalimetric titration using tributyrin as substrate. A fixed amount of enzyme (from 0.125 to 3 mg/ml) was incubated with the MoAb at different concentrations. Incubations were performed in cell culture medium or in PBS for 2 h at 4 °C. The residual activity of lipase–MoAb complex was measured using the standard method of tributyrin hydrolysis (alkalimetric final titration). The assay mixture, containing 2 ml of buffer (20 mM phosphate buffer, pH 7.4), 0.5 ml of tributyrin and lipase–MoAb complex solution, was incubated at 37 °C under magnetic stirring (300 rpm) for 30 min. The reaction mixture was stopped with 2.5 ml of acetone/ethanol mixture 1:1 (v/v) and titrated with 0.01 M NaOH in the presence of phenolphthalein as indicator using an automatic burette.

Native electrophoresis was performed using a combination of Phastsystem with ready PhastGel media with 8–25% gradient in polyacrylamide gel. The run was performed in three steps up to 229Avh. The α-naphtyl acetate in *N*,*N*-dimethylformamide and Fast Garnet GBC were used for the specific esterasic activity staining [8]. After the run, the gels were equilibrated for 30 min at 4 °C in buffer (MES 20 mM, pH 6.4). 10 mg of α-naphtyl acetate were solubilized in 0.4 ml of *N*,*N*-dimethylformamide and added to a Fast Garnet GBC solution (10 mg in 20 ml MES). The staining mixture was added to gel, the spots appeared in 10–30 min.

Proteins were separated on 10% SDS–PAGE and transferred electrophoretically to nitrocellulose membrane. After blocking o/n with 5% non-fat dried milk in PBS, the membrane was incubated with the MoAbs for 2 h and then for 1 h with peroxidase conjugated goat anti mouse Ig. The membrane was then developed using the enhanced chemioluminescence Western blotting detection reagents and Hyperfilm ECL Autoradiography Film.

4. Conclusions

We have described the generation and characterization of two MoAbs of the IgG_1 isotype directed against distinct epitopes of CRL. These MoAbs represent novel reagents since up to now only polyclonal antibodies against CRL have been described [13]. Both MoAbs are specie-specific recognizing all lipase isoform tested (Lip1, 2 and 3). Moreover the binding of the immunoglobulin do not affect either the lypolitic or the esterasic enzymatic activity suggesting the possible use of these reagents in a biotechnological setting and on-line monitoring of fermentative CRLs production.

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