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Journal of Molecular Catalysis B: Enzymatic 3 (1997) 51–54

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Effect of mutation in non-consensus sequence Thr–X–Ser–X–Gly of *Candida antarctica* lipase B on lipase specificity, specific activity and thermostability

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Received 17 August 1996; accepted 13 September 1996

Abstract

Non-consensus residue threonine in *Candida antarctica* B lipase was exchanged with glycine residue using site specific mutation. The effect of the mutation on the thermostability was investigated by measuring residual activity after heat treatment of the lipase and the mutant. A significant increase in thermostability was found for the mutant lipase. Specific activity of the mutant lipase was determined using tributyrin as substrate which showed a twofold decrease in specific activity. To investigate the effect of mutation on the specificity and activity in ester synthesis, both the mutant lipase and the native lipases were immobilized on a solid support. Ester synthesis using decanol as alcohol with three different fatty acids was carried out. The activity and specificity of the mutant lipase was unaltered in the ester synthesis as compared with the native lipase.

1. Introduction

The crystal structures of several microbial lipases have been published [1–5]. One common feature of all these lipases is an active site triad consisting of a serine as the nucleophile, histidine as base and either aspartic acid or glutamic acid as the acidic residue. Microbial lipases and mammalian lipases show no obvious similarities in their primary structures. Within the group of microbial lipases, a large sequence variety is found. One common feature in mammalian and microbial lipases is that almost all contain the consensus sequence Gly–X–Ser–X–Gly around the active serine [6,7].

Another interesting group of enzymes is the members of the prolyl oligopeptidase family which contain the same consensus sequence, and their catalytic triad might be covered with a flap similar to those found in lipases [8]. In the carboxypeptidase group the second conserved glycine residue is changed to alanine, i.e. G–X–S–X–A [8], *Bacillus subtilis* lipase was shown to deviate from the consensus sequence [9]. We have previously shown that *Candida antarctica* produces two different lipases named A and B [10].

Lipase A was non-specific and the most thermostable lipase, but lipase B was more interest-

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PII S1381-1177(96)00036-7

ing for industrial applications such as ester synthesis and production of peracids [11,12]. Lipase B from *Candida antarctica* has been cloned and sequenced. The sequence and crystal structure of lipase B was recently reported [4]. It has a threonine residue instead of a glycine as the first residue in the consensus sequence. Its specific activity is only 500 U/mg using tributyrin as substrate. In order to investigate if the threonine residue plays any role for specific activity, specificity and stability, we mutated it back into a glycine.

2. Materials and methods

2.1. Cloning and expression

The gene for *Candida antarctica* lipase B has been cloned and sequenced. Expression of the gene has been achieved in *Aspergillus oryzae* by co-transformation with an expression plasmid and a marker plasmid carrying the acetamidase gene (amdS) from *Aspergillus nidulans* essentially as previously described for the *Rhizomucor miehei* lipase [13].

2.2. Site-directed mutagenesis

The approach used for introducing mutations into the lipase gene has been described previously [14]. It involves the 3-step generation of a PCR (polymerase chain reaction) fragment containing the desired mutation introduced by using a chemically synthesized DNA-strand as one of the primers in the PCR-reactions. From the PCR generated fragment, a DNA fragment carrying the mutation can be isolated by cleavage with restriction enzymes and re-inserted into the expression plasmid. Using that method, the threonine in position 103 in the mature *Candida antarctica* lipase B was exchanged by a glycine by shifting the codon from ACC to GGT. The primer used for the mutagenesis had the sequence 5′-CTTCCCGTGCTTGGTTGGTCCCAGGGT-3′. The mutation as well as the DNA fragment generated by PCR was verified by DNA sequencing.

2.3. Assay of lipase activity

Lipase activity was assayed using tributyrin as substrate and gum-arabic as emulsifier at 30°C using the pH-stat method as described previously [15].

2.4. Purity of lipases

Cloned *Candida antarctica* lipase B and mutant lipase were purified by conventional ion exchange and hydrophobic chromatography. Both the lipases were checked for homogeneity by SDS–PAGE.

2.5. Thermo-inactivation in solution

The protein concentration of the *Candida antarctica* lipase B and the mutant lipase was adjusted to 1 mg/ml in 50 mM Tris acetate buffer (pH 7). Both the lipases were pre-incubated at 60°C and aliquots were withdrawn at different time intervals, diluted in water and assayed for lipase activity.

2.6. Immobilization

A stirred solution of mutant lipase T103G (2 mg/ml) at pH 7.0 was exposed to a polystyrene matrix (Accurel EP100, 40 mg/ml) for 4 h at room temperature. A load of 50 mg/g carrier was obtained. *Candida antarctica* lipase B was immobilized using the same procedure.

2.7. Ester synthesis

To a stirred mixture of decanol (117 mg, 0.75 mmol) and either dodecanoic acid, octadecenoic acid, or 9-*cis*-octadecenoic acid (0.75 mmol, 150, 213 and 212 mg, respectively) at 60°C was added either 10 mg immobilized mutant lipase T103G or immobilized native *Candida antarctica* lipase B. After 2 h the ester formation was

Table 1
p*I* and specific activity of wildtype and mutant lipases. The activity assay was carried out with tributyrine as substrate

	MW by SDS–PAGE	p*I*	Spec. act. Lu/mg
C. antarctica B	35 kD	6.0	500
mutant T103G	35 kD	6.0	260

Table 2
Synthesis of ester by exposing decanol to various fatty acids with either immobilized T103G or native B lipase

Lipase	Dodecanoic	Octadecenoic	9-*cis*-Octadecenoic acid
C. antarctica B	72%	72%	71%
mutant T103G	68%	72%	70%

monitored by NMR spectroscopy (using $CDCl_3$ as the solvent with tetramethyl silane (TMS) as internal reference employing a Bruker acp 300 spectrometer).

3. Results

3.1. Specific activity and pI

Table 1 summarizes the molecular weight, p*I* and specific activity of *Candida antarctica* lipase B and of the mutant lipase. The molecular weight and isoelectric points are identical for both the native and mutant lipases. The specific activity of the mutant lipase was reduced to almost half of the activity of the native enzyme.

3.2. Thermostability

Fig. 1 shows that native lipase had lost half of its activity in 6 min at 60°C whereas the mutant T103G lipase maintained 50% activity for up to 25 min. This shows a pronounced increase in thermostability of the mutant lipase.

3.3. Effect of the mutation on ester synthesis

The activity of T103G in ester synthesis was evaluated by exposing decanol to various fatty acids using either immobilized T103G lipase or *Candida antarctica* lipase B (the native enzyme) as catalyst. Almost identical conversions were obtained in all experiments indicating T103G to have a specificity and activity comparable to the native enzyme, as shown in Table 2.

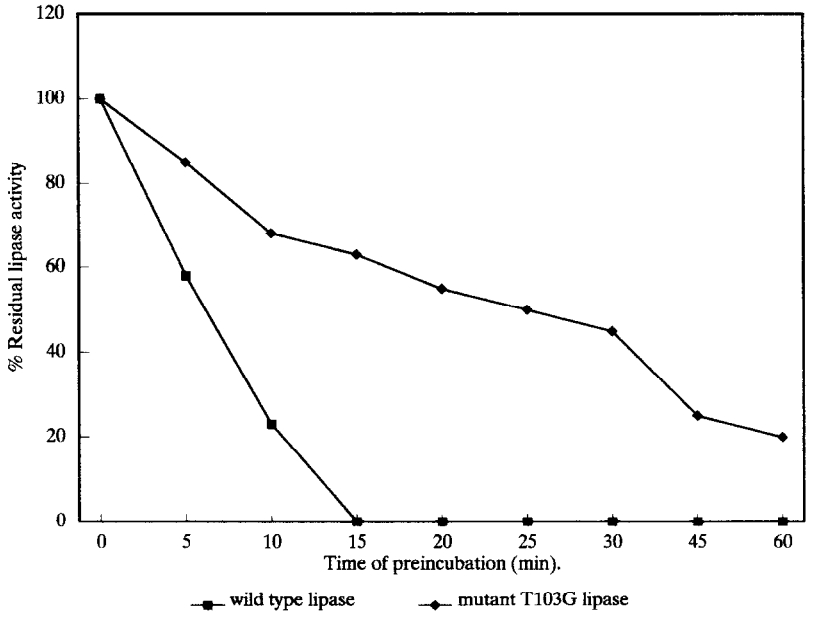

Fig. 1. Comparison of activity of wild type and mutant T103G of *C. antarctica* B lipase. Thermostability. The figure shows the difference in decay of lipase activity of the native and mutant lipases upon exposure to heat in solution.

4. Discussion

In all the known structures of lipases, the consensus sequence is located in a nucleophilic elbow formed by a β-sheet and an α-helix [16,17]. In the *Rhizomucor miehei* lipase the first glycine in the consensus sequence is deeply buried, both in open or closed conformation [18], in *Candida antarctica* B lipase it is a threonine in the equivalent position. A structural comparison of the nucleophilic elbow in *Rhizomucor miehei* and *Candida antarctica* B lipases show that the α-helix in the nucleophilic elbow of *Candida antarctica* lipase B is slightly more bent away from the β-strand [4]. The threonine 103 sidechain are in close contact with the opposite α-helix.

It can be suggested that the increase in thermostability of *Candida antarctica* lipase B mutant arises from increased number of internal contacts between the α-helix and the β-strand. If the threonine 103 placed in *Rhizomucor miehei* at the glycine position it would push the α-helix away from the β-strand. Replacing glycine by a threonine in the *Rhizomucor miehei* lipase would definitely introduce structural changes in this highly homologous region. Removing the threonine in the *Candida antarctica* lipase B and assuming that the overall structure is more or less intact, an empty space is probably formed, which would leave room for additional internal water atoms. Alternatively the structural elements will adopt to a closer and better contact now that the sidechain of residue 103 has been removed. Whether the increased stability is a result of additional water in the free space created by the mutation, or from structural adaptation of new and better internal contact in the mutant structure, must await the solution of the X-ray structure of the mutant lipase.

References

[1] L. Brady, A.M. Brzozowski, Z. Derwenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenberg, L. Christensen, B. Huge-Jensen, L. Norskov, L. Thim and U. Menge, Nature, 343 (1990) 767.

[2] J.D. Schrag, Y. Li, S. Wu and M. Cygler, Nature, 351 (1991) 761.

[3] M.E.M. Nobel, A. Cleasby, L.N. Johnson, M.R. Egmond and L.G.J. Frenken, FEBS Lett., 331 (1993) 123.

[4] J. Uppenberg, M.T. Hansen, S. Patkar and A. Jones, Structure, 2 (1994) 293.

[5] O. Misset, K.E. Jaeger, U. Winkler, C. Colson, K. Schanck, E. Lesuisse, V. Dartois and M. Blaauw, Protein Eng., 7 (1994) 523.

[6] A. Svendsen, in P. Woolley and S.B. Petersen (Eds.), Lipases, Cambridge University Press, Cambridge, 1994, Chap. 1, p. 1.

[7] S.B. Petersen and F. Drabløs in P. Woolley and S. Petersen (Eds.), Lipases, Cambridge University Press, Cambridge, 1994, Chap. 2, p. 23.

[8] L. Polgar, FEBS Lett., 311 (1992) 281.

[9] V. Dartois, A. Baulard, K. Schanck and C. Colson, Biochim. Biophys. Acta, 1131 (1992) 253.

[10] S.A. Patkar, F. Bjørkling, M. Zundel, M. Schulein, A. Svendsen, H.P. Heldt-Hansen and E. Gormsen, Indian J. Chem., 32B (1993) 76.

[11] H.P. Heldt-Hansen, M. Ishi, S.A. Patkar, T.T. Hansen and P. Eigtved, in J.R. Whitaker and P.E. Sonnet (Eds.), Biocatalysis, Agricultural Biotechnology, ACS Symposium Series, Vol. 389, 1988, p. 158.

[12] F. Bjørkling, S.E. Godtfredsen, O. Kirk, S.A. Patkar and O. Andreasen, in S. Servi (Ed.), Microbial Reagents in Organic Synthesis, Kluwer, Dordrecht, 1992, p. 249.

[13] T. Christensen, H. Woeldike, E. Boel, S.B. Mortensen, K. Hjortshoej, L. Thim and M.T. Hansen, Biotechnology, 6 (1988) 1419.

[14] R.M. Nelson and G.C. Long, Anal. Biochem., 180 (1989) 147.

[15] M. Barfoed, K. Borch, H. Lund and S.A. Patkar, in W.J.J. van der Tweel, A. Harder and R.M. Buitelaar (Eds.), Stability and Stabilization of Enzymes, Studies in Organic Chemistry, Vol. 47, 1992, p. 181.

[16] D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Verschueren and A. Goldman, Protein Eng., 5 (1992) 197.

[17] S. Brenner, Nature, 334 (1988) 528.

[18] A.M. Brzozowski, U. Derwenda, Z.S. Derwenda, G. Dodson, D.M. Lawson, J.P. Turkenberg, F. Bjørkling, B. Huge-Jensen S.A. Patkar and L. Thim, Nature, 351 (1991) 491.