Bacillus thermocatenulatus lipase: a thermoalkalophilic lipase with interesting properties
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Lipases (triacylglycerol lipase EC 3.1.1.3) catalyse hydrolysis and transesterification of triacylglycerols. Industrial applications of lipases include the production of non-esterified fatty acids, interesterification of oils and fats and synthesis of esters and peptides.

Proteins from thermophiles generally exhibit high thermodynamic stability, both at elevated temperatures and in organic solvents [1–4], important properties for the industrial application of enzymes [5–7]. We have focused our attention on lipases from thermophiles to further enhance thermostability and to gain insight into the structural requirements for thermodynamic stability.

Molecular cloning and sequence analysis
The thermophile B. thermocatenulatus produces two lipases of different size: 16 kDa and 43 kDa [8]. The 16 kDa lipase was purified directly from the culture broth of B. thermocatenulatus [9]. However, generation of an expression library in pUC18 by partial Sau3A digestion of genomic DNA of B. thermocatenulatus yielded a clone coding for a higher-molecular-mass (43 kDa) lipase [10]. A single open reading frame of 1251 bp was found to code for the enzyme. Sequences upstream of the lipase gene showed a promoter sequence that directed the expression of the lipase at a low level (600 units/g) in Escherichia coli DH5α.

The deduced sequence of the preprotein contains 417 amino acids and corresponds to a molecular mass of 46.23 kDa. The mature lipase contains 388 amino acids, which corresponds to a molecular mass of 43.09 kDa. A comparison of the deduced amino acid sequence with that of several lipases in the region of the catalytic triad His, Ser, Asp (Glu) and around the oxyanion hole allowed us to determine that Ser-113, Asp-317 and His-315 are the residues forming the catalytic triad and that the oxyanion hole is formed by the residues around His-14. However, no significant homology apart from the region around the nucleophile serine was found with most other lipases and esterases in the EMBL and SwissProt databases. However, a significant homology of 30–35% with the mature lipases of Staphylococcus hyicus, Staphylococcus aureus and Staphylococcus epidermidis was found [11–14]. Like the lipases from Bacillus subtilis and Bacillus pumilus [15,16], an Ala replaces the first Gly in the pentapeptide Gly-X-Ser-X-Gly, which is conserved among microbial and mammalian lipases. This replacement might be a common feature of Bacillus lipases.

Overexpression in E. coli
The low expression level under the control of its native promoter made it necessary to develop an efficient expression system for lipase production. Large amounts of protein are required for crystallographic studies, biochemical characterization and biotechnological applications. Based on a
structure model, mutagenesis could be applied to the lipase, improving the enzymic properties in the desired direction for special applications.

For this purpose, we subcloned the lipase gene in the *E. coli* expression vector pCYTEXP1 [17] downstream of the strong temperature-inducible λ promoter PL. Two general expression strategies were developed: (a) expression of mature lipase in the cytoplasm of *E. coli* (expression vector pT-BTL) and (b) secretion of lipase in the periplasm of *E. coli* by the use of either the original lipase leader peptide (expression vector pT-preBTL) or the OmpA signal sequence (expression vector pT-OmpABTL) (M. L. Rua, H. Atoni, C. Schmidt-Dannert and R. D. Schmid, unpublished work).

In Table 1 the expression levels obtained with the three expression vectors in different *E. coli* host cells after 3 h of induction are shown. These expression levels represent a 12–50-fold improvement over the lipase expression under the control of the native promoter. Introduction of the OmpA presequence upstream of the lipase gene resulted in a 3-fold increase in lipase expression when compared with pT-BTL and pT-preBTL. A further 20-fold increase in the expression of soluble lipase was obtained when pT-OmpABTL was introduced in *E. coli* JM105 (660 000 units/g of cells), representing an expression level of about 30% of the total cell protein. However, most of the expressed lipase remained insoluble but active in the pellet after cell disruption.

Lipase produced by pT-preBTL was only partially processed whereas lipase produced by pT-OmpA-BTL was not at all processed by the *E. coli* host cell. With both pT-preBTL and pT-OmpABTL we tried to increase the amount of mature lipase by using conditions for cultivation and expression that should reduce the rate at which newly synthesized proteins are released by the protein-synthesis machinery into the cell cytoplasm [19,20]. However, only a small increase in the amount of mature lipase in the case of pT-preBTL and no influence on the processing of the OmpA-lipase was found. In the case of pT-OmpABTL it is likely that the cell machinery responsible for the processing of the protein was simply overloaded by such a high level of lipase production.

### Table I

<table>
<thead>
<tr>
<th>Expression vector</th>
<th><em>E. coli</em> host cell</th>
<th>Expression level (units/g of cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT-BTL</td>
<td>BL32I</td>
<td>7 000</td>
</tr>
<tr>
<td>pT-preBTL</td>
<td>BL32I</td>
<td>9 000</td>
</tr>
<tr>
<td>pT-OmpABTL</td>
<td>BL32I</td>
<td>30 000*</td>
</tr>
<tr>
<td></td>
<td>DH5α</td>
<td>30 000*</td>
</tr>
<tr>
<td></td>
<td>JM105</td>
<td>660 000*</td>
</tr>
</tbody>
</table>

*Soluble lipase in the supernatant after cell breakage.

Large-scale production and purification

For crystallization it must be ensured that only the mature lipase form is present in the protein solution. Hence *E. coli* BL32I pT-BTL was chosen for cultivation in a 100-litre fermenter (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work). Lipase, which is located in the cytoplasm of *E. coli* cells, was purified to homogeneity after cell disruption in a three-step procedure, including chromatography on butyl-Sepharose as the key step followed by ultrafiltration and gel filtration. This purification procedure resulted in a highly active and pure lipase with a yield of 32% and a specific activity of 54 887 units/mg. Table 2 summarizes the results of the purification (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work).

In order to utilize the large amount of lipase expressed by *E. coli* JM105 pT-OmpABTL for applications other than crystallization, it was necessary to cleave the OmpA leader sequence.
Table 2

Summary of the purification of recombinant lipase from B. thermocatenulatus produced with E. coli BL321 pT-BTL2

Specific activity was measured using a pH-stat assay with tributyrin as substrate (60 °C and pH 7.5).

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell breakage</td>
<td>942 270</td>
<td>1215</td>
<td>483</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Butyl-Sepharose</td>
<td>481 815</td>
<td>39</td>
<td>12 354</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>403 875</td>
<td>15.8</td>
<td>25 562</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>296 391</td>
<td>5.4</td>
<td>54 887</td>
<td>125</td>
<td>32</td>
</tr>
</tbody>
</table>

from the lipase. For this, we developed a rapid purification procedure including (a) detergent extraction of insoluble but active OmpA-lipase from the pellet obtained after cell disruption, (b) cleavage of the OmpA leader sequence from the extracted lipase by proteinase K treatment, (c) ultrafiltration and (d) a final purification by gel filtration (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work). By this procedure we obtained a pure lipase with a total yield of 50% and a specific activity of 36 000 units/mg.

Physicochemical properties
The pure and highly active lipase overexpressed in E. coli had a pI of 7.2 and a molecular mass of 40 kDa on SDS/PAGE. The specific activity of the overexpressed lipase is 320–1675 times higher than that of the 16 kDa lipase previously isolated from B. thermocatenulatus and other small Bacillus lipases. In addition, most lipases have specific activities below 10 000 units/mg.

As expected for a thermophilic protein, the lipase reaches maximum activity between 55 and 75 °C depending on the substrate. With triolein as substrate, maximal activity was found at 75 °C and with tributyrin at 60 °C. Also, the lipase is thermostable, like most lipases from Bacillus and Pseudomonas species. The enzyme was stable up to 50 °C when incubated for 30 min at pH 9.0 (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work).

The pH–activity of the lipase at 60 °C depends on the substrate. With triolein, maximum activity was found at pH 9.0, decreasing rapidly as pH decreased so that activity was no longer detected at pH 6.0. With tributyrin, on the other hand, a maximum was reached at pH 8.0 and, in contrast with triolein, 70% residual activity was detected at pH 6.0. After 14 h of incubation at 30 °C, the enzyme was stable within the pH range 9.0–11.0. Thus, unlike the small 16 kDa lipase of B. thermocatenulatus and most other lipases, the overexpressed 43 kDa lipase is a thermoalkalophilic enzyme.

Most detergents enhanced the activity, with the increase ranging from 40% (CHAPS and octylglucoside) to 80% (Triton X-100 and cholate), when added to the enzyme solution and did not inactivate the enzyme after 1.5 h of incubation at 30 °C. But the addition of SDS, Lubrol PX, Tween 20 and Tween 80 immediately inactivated the enzyme (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work).

The lipase showed only a 20% decrease in activity when incubated in the presence of 30% (v/v) methanol, propanol or acetone for 1 h at 30 °C (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work). After prolonged incubation for 4 days at room temperature in the presence of 30% (v/v) propanol, no further decrease in activity was detected, indicating that the enzyme is exceptionally stable in the presence of organic solvents.

The lipase favours tributyrin as substrate, as do lipases from S. hyicus and S. epidermidis. The sequence similarities among these three may be responsible for this similar substrate preference. The activity on longer-chain triacylglycerols was between 40% (C₆ or C₈) and 20% (C₁₂ to C₁₆) that of the activity on tributyrin. Triacetin (C₃) was a poor substrate being hardly hydrolysed at all (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work).

Some further similarities exist between the overexpressed lipase and a purified lipase from a thermophile Bacillus sp. 398 strain [22]. This lipase, like the 43 kDa lipase of B. thermocatenulatus, is a large lipase of 50 kDa, with a high...
specific activity (25 300 units/mg). It, too, reaches maximum activity at 60–70 °C.

**Studies on the aggregation behaviour**

The tendency to form aggregates is a well-known property of several lipases [23–25]. Both lipases (the 16 and 43 kDa enzymes) form aggregates, as can be observed during native PAGE or gel filtration.

Dramatic changes in the aggregation behaviour of the overexpressed lipase and hence its specific activity were observed under different conditions. Gel filtration on a TSK G3000 (Tosohaas) column under various conditions was used to study the aggregation behaviour of the lipase (Table 3) (M. L. Rua, C. Schmidt-Dannert, S. Wahl, A. Sprauer and R. D. Schmid, unpublished work). Pure lipase obtained after hydrophobic chromatography on butyl-Sepharose changed its aggregation state in response to the running conditions. In the absence of organic solvents or detergents the lipase exists in an aggregated form. The presence of 30% (v/v) propanol in the running buffer during gel filtration led to total disaggregation of the lipase aggregates. In the presence of 1% (w/v) cholate, where a molecular mass of 72 kDa for the lipase was detected after gel filtration, which does not correspond to the molecular mass of the monomer, it is likely that the lipase is associated with cholate molecules. However, detergents other than cholate, such as 1% Brij, failed to dissociate the molecular aggregates of the lipase completely. It is not clear whether the lipase interacts with bile salts in a specific way, since only bile salts (cholate, deoxycholate, taurocholate) were able to elute the lipase from the butyl-Sepharose column during purification. Other detergents (octylglucoside, Brij, Triton X-100), organic solvents (propanol, acetone or methanol) and guanidine, all compounds that are effective at weakening hydrophobic interactions, were ineffective.

The incubation of lipase fractions with different aggregation states obtained after gel filtration in the presence or absence of 1% cholate for 5 min, after which the activity was checked with triolein as substrate, clearly showed that the active site of the lipase molecule is involved in the aggregation (Table 3). Under those conditions where monomers of the lipase existed in the solution [30% (v/v) propanol], the activity did not change on incubation with the detergent. For this reason, the ratio between the specific activities measured before and after incubation with detergent was 1. In contrast, the activity of the lipase with the highest molecular mass (>500 kDa) after gel filtration increased by a factor of 6.8 after incubation with 1% (w/v) cholate.

Nevertheless, the effect of aggregation was strongly dependent on the substrate (tributyrin or triolein) employed for the activity assay and the temperature during the assay (30 °C or 60 °C). The tributyrin-hydrolysing activity of aggregated lipase (>500 kDa) was not changed at 30 and 60 °C by incubation with 1% cholate, but a strong enhancement of the triolein-hydrolysing activity was observed, especially at 30 °C. One possible explanation could be that, during tributyrin hydrolysis, monobutyrin is produced.

### Table 3

Comparison of the estimated molecular mass of recombinant lipase obtained after gel-filtration chromatography under several running conditions and the ratio between the specific activity (units/mg) measured before (−) and after (+) 5 min of incubation with 1% cholate

Specific activity was measured by a pH-stat assay with triolein as substrate (30 °C and pH 8.5).

<table>
<thead>
<tr>
<th>Running conditions</th>
<th>Estimated molecular mass (kDa)</th>
<th>+/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris/HCl buffer, pH 7.5</td>
<td>&gt;500</td>
<td>6.8</td>
</tr>
<tr>
<td>50 mM Tris/HCl buffer, pH 7.5, 0.1% (w/v) cholate</td>
<td>235</td>
<td>4.1</td>
</tr>
<tr>
<td>50 mM Tris/HCl buffer, pH 7.5, 1% (w/v) cholate</td>
<td>72</td>
<td>1.9</td>
</tr>
<tr>
<td>50 mM Tris/HCl buffer, pH 7.5, 30% (v/v) propanol</td>
<td>44</td>
<td>1.0</td>
</tr>
</tbody>
</table>
which might act as a surfactant (in contrast with the water-insoluble long-chain mono-olein) breaking down the lipase aggregates. A more specific effect of tributyrin cannot be excluded.

**Conclusion**

The recombinant lipase described here proved to be a very stable enzyme, with optimal activity at elevated temperatures and at alkaline pH, which are promising properties for biotechnological applications. This large 43 kDa lipase, which was previously not detected during cultivation of *B. thermocatenulatus*, is different from most other lipases of *Bacillus*. Moreover, this lipase shows homology to lipases from *Staphylococcus* strains. Until now no structure for such a large prokaryotic lipase has been available. Therefore we developed an efficient expression system and a simple purification procedure to allow us to produce the large amounts of pure enzyme needed for crystallization, which is now in progress. In addition, further comparisons with lipases from *Staphylococcus*, including the expression of the *B. thermocatenulatus* lipase in *Staphylococcus carnosus*, are in progress.

18. Reference deleted
21. Reference deleted

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