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ENZYMES WITH A LOW MOLECULAR WEIGHT

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Abstract.
Two esterase enzymes have been isolated, one from *Candida lipolytica* and one from *Bacillus stearothermophilus*, which are characterised by an unusually small molecular weight. The *Candida* enzyme is 5.7 kDa, with 56 amino acid residues and the *Bacillus* enzyme is 1.57 kDa, with only 17 residues (1).
In both cases the catalytic activity appears to depend on a bound metal ion, as shown by dialysis against chelating agents, ion replacement and inhibition by metal complexing agents. Specific activities are similar to reported esterase activities.
The *Candida* esterase has a temperature optimum of 28^0 , as might be expected from a mesophilic organism, but it has a half-life of 2 hours at 50^0 . The esterase from *B.stearothermophilus* is thermophilic, but whereas the optimum growth temperature is 55^0 the enzyme optimum is about 120^0.
Both enzymes exhibit some substrate specificity. The *Bacillus* enzyme has no particular specificity towards the chain length of the substrate, but shows a marked activity towards the 2-position of triglycerides. The *Candida* enzyme shows both chain length specificity (optimum at butyl esters), as well as specificity towards the 1-position.

Introduction

The size of enzymes has been the subject of debate for many years; the question is often posed in the form “Why are enzymes large?” The text-book answer is usually given in terms of bond energies; biological systems operate with two broad classes of chemical bond, firstly “weak” bonds, such as van der Waals Forces (1 kcal/mole), hydrogen bonds (3 - 7 kcal/mole), ionic bonds (5 kcal/mole) and hydrophobic

interactions (-1 to -3 kcal/mole) and secondly “strong” covalent bonds (~20 kcal/mole) .

The weak bonds are responsible for virtually all the higher orders of biochemical structure, as well as most biochemical interactions that demand a high degree of stereochemical specificity. As these bonds are no more than an order of magnitude greater than the thermal energy of the environment such weak interactions are readily disrupted by the kinetic energy present in living systems. Covalent bonds, on the other hand, are not readily broken at physiological temperatures, enzyme - catalysed reactions are required for such bonds to be rapidly made and ruptured. The rate at which an enzyme catalyses a chemical reaction is probably dependent on the rapidity of a reversible conformational change induced by interaction with a substrate; such conformational changes involve breaking and forming many weak bonds throughout the protein, with the result that a covalent bond is cleaved.

Because weak bonds can be broken by the kinetic energy released during the binding of substrates and modulators to enzymes so they can be broken by the addition of kinetic energy in, for example, the increase in environmental temperature. These changes may reduce the catalytic function, or the different tertiary and quaternary structures at the new temperature might have a enhanced functional properties. Thus size, function and thermostability are closely linked in proteins.

As the enzyme molecule becomes smaller the number of hydrogen bonds etc. falls, so that less catalytic activity but more stability might be expected.

The purpose of this paper is to establish that the size range of naturally occurring proteins with catalytic activity is greater than hitherto thought, secondly that such enzymes exhibit thermal properties that are both consistent with their size and are adapted to the rigorous extracellular environment, and thirdly that their mechanism of action results in substrate and product specificity.

There have been a few reports in the literature in which an enzyme activity has been attributed to proteins with a molecular weight of less than 10 kDa (“microenzymes”)(2). A lipase from bovine milk slime with an apparent molecular weight of 7 kDa was reported by Chandan & Shahani (3). The molecular weight was

determined by sedimentation velocity and osmotic pressure methods, and the activity exhibited a pH optimum of 9.2 and 37^0.

A rennin from an unidentified thermophilic actinomycete, isolated from soil near Beer Sheva, was found to have a molecular weight of 10.5 kDa, based on analytical ultra-centrifugation and SDS electrophoresis (4). The amino acid composition was determined and the protein was found to have 78 residues, of which 9 were proline, giving a molecular weight of 9.7 kDa. The temperature optimum was 75^0, and it required calcium ions for activity. Limited proteolytic activity against insulin was noted.

Another small proteolytic enzyme was reported by Steele et al. (5) isolated from a novel spiral bacterium, *Kurthia spiroforme*. This gram-positive bacterium grew at neutral pH in a thermal spring, but exhibited a wide range of growth temperatures and pH values, ranging from 4^0 to 47^0 and a pH range from 7 to 11.5 with an optimum of 30^0 and pH 10.5. The extracellular protease was alkaline-stable and had an optimum temperature of 60^0 and optimum pH of 11.

An amylase from *Bacillus caldolyticus* (6) was shown to have active subunits of less than 10 Kda, which associate in the presence of calcium ions to give the classic thermophilic amylase active at 70^0. The subunits of the thermophilic amylase exist when calcium is absent or low in concentration and are not thermophilic but are still thermostable, showing activity up to 40^0, but are stable up to 60^0, as shown by the recovery of activity when the temperature is reduced, or when calcium is added at a higher temperature.

We have described two extracellular peptides with esterase activity, one produced by a strain of *Candida lipolytica* (CMI 92,743) (7) and one from *Bacillus stearothermophilus* (1). The esterase from the *Candida* species had a molecular weight of 5 kDa ± 500 as determined by both Sephadex G-100 gel filtration and SDS-polyacrylamide electrophoresis. The enzyme contained 56 amino acid residues, 13 of which were proline, giving a molecular weight of 5717 kDa. The *Bacillus* esterase was much smaller, with 15 amino acid residues, none of which was proline, giving a molecular weight of 1556 kDa. The composition of the two esterases is shown in Table 1.

One feature of the Candida esterase is the high percentage of proline, a feature also noted with the rennin from a thermophilic actinomycete, although at 23.7 mole%

Amino acid	***Candida***	***Bacillus***
Ala	5	1
Asp	3	3
Arg	1	1
Cys	1	-
Glu	8	1
Gly	8	2
His	3	2
Ileu	1	-
Leu	3	-
Lys	1	-
Pro	13	-
Phe	1	-
Ser	1	4
Thr	2	1
Tyr	1	-
Val	4	-
Total	56	15

Table 1 Amino acid composition of the extracellular esterases from *Candida lipolytica* and *Bacillus stearothermophilus*. Amino acid analysis was carried out on an Applied Biosystems 420H Amino acid Analyser using phenylthiocarbamyl derivitisation. Calibration used an internal standard of norleucine.

the esterase has twice the proline content.

Proline has been associated with increased protein thermostability (8, 9) on the basis of the different thermostability of five *Bacillus* oligo-1,6- glucosidases. Comparisons were made of aminoacid composition and structural parameters and from the analysis, in conjunction with the strong site specificity of proline residues for β-turns (10, 11, 12) it was proposed that enhanced stability could be gained by increasing the frequency of proline occurrence at β-turns and the total number of hydrophobic residues (8) This appeared to be given support by Matthews *et al* (13) where the thermostability of bacteriophage lysozyme was increased by replacing alanine with

proline at position 82 of a β-turn so as to decrease the backbone entropy of unfolding. Other results have correlated increased proline with increase thermostability with different enzymes (14, 15, 16) The correlation with both pullulanases and oligo-1.6-glucosidases between proline percentage and T_m (the temperature at which the enzyme was half inactivated in 10 minutes at pH6.8) was linear between 3% and 9% proline and 45^0 to 98^0. The *Candida* esterase does not fall on that line, although its T_m, at 70^0 is higher than expected for a mesophilic organism. The rennin (3) similarly does not coincide with the *Bacillus* data, but its stability (T_m 75^0) is greater than the optimum growth temperature (52^0).

Mode of Action:

The two esterases show different behaviour towards a range of inhibitors (Table 2)

Addition	***Candida lipolytica***	***Bacillus stearothermophilus***
None	100	100
NaN_3	35	90
KCN	29	85
p-chloro-mercuribenzoate	29	79
$CuSO_4$	100	0
$FeSO_4$	100	80
1,10 phenanthroline	20	113
EDTA	30	89

Table 2. The effect of inhibitors on the activity of the esterases from *Candida lipolytica* and *Bacillus stearothermophilus*.The additions were made at 5mM.

The *Candida* esterase shows a pattern of inhibition consistent with iron chelation, with the cytochrome oxidase inhibitor azide, p-chloro-mercuribenzoate and 1,10 phenanthroline being potent inhibitors, and general metal chelators such as EDTA

also effective. The *Bacillus* esterase shows slight inhibition with some of these inhibitors, but with 1,10 phenanthroline shows slight activation. The most striking inhibitor of the *Bacillus* enzyme was copper sulphate which had an effect considerably greater than other inorganic salts, for example ferrous sulphate. Inorganic salts are known to weaken electrostatic forces within the protein molecule (17), but the complete inhibition could not be attributed to this. Cu^{2+} is known to form complexes with amino and amide groups, as in the classic Biuret test for proteins.

If the enzymes are dialysed against EDTA then a loss of activity is observed in both cases (Fig 1). The rate of activity loss is similar in both cases although the temperature at which the *Candida* enzyme (28^0) and the *Bacillus* enzyme (70^0) were treated was different.

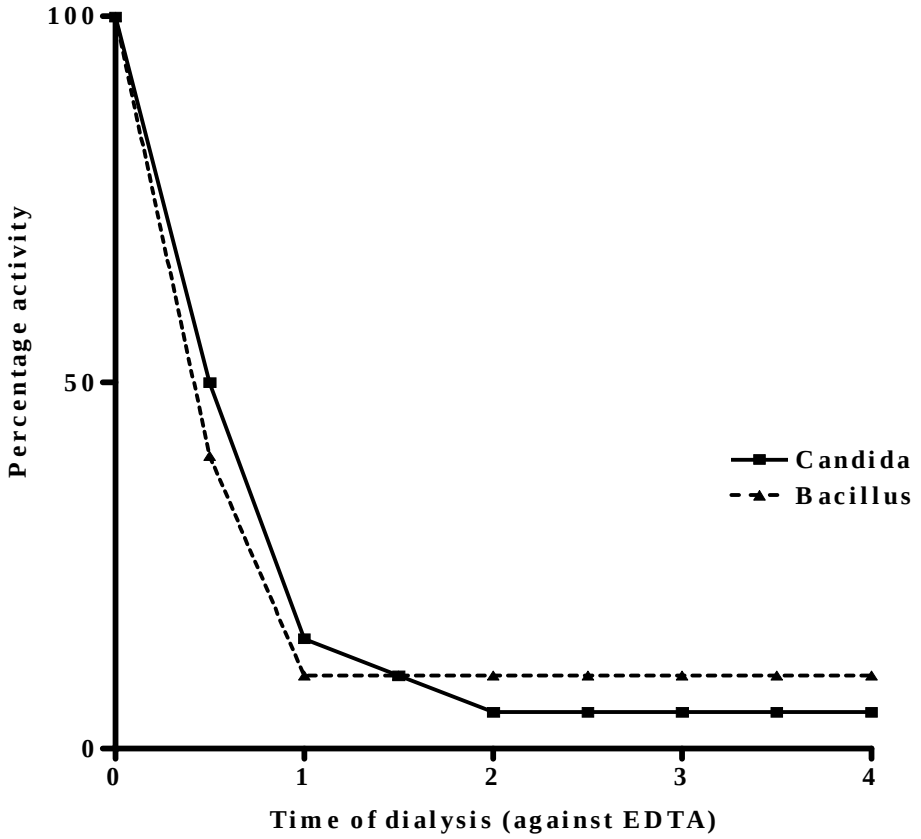

Figure 1. The effect of dialysis on esterase activity. The enzyme preparations were dialysed against 1mM EDTA at 70^0 in the case of *Bacillus* and 28^0 in the case of *Candida* for four hours. The results are expressed as a percentage of the starting

activity as determined by fluorometric assay using fluorescein dibutyrate at 10^{-7}M as substrate.

Attempts to restore the activity were made with a number of cations, added at 5 mM to the dialysed solution. For the esterase from *Candida lipolytica* 90% of the original activity was restored by ferric ions, other ions such as zinc, ferrous ions, nickel and copper restored about 20%, while monovalent ions, calcium and magnesium either did not have any effect or further reduced the residual activity.

For the *Bacillus* esterase the only ion found with any significant effect was Fe^{3+}, but only 40% activity was restored. When dialysis was carried out against water instead of EDTA the activity was lost more slowly, but 80% of the activity could be restored by mixing the dialysed enzyme with the dialysis solution, unlike ferric ions where again 40% activity was restored.

Effect of pH.

The two esterases showed quite distinct pH profiles, the *Candida* esterase showed a flat profile until pH values below 2, while the *Bacillus* enzyme showed an optimum at pH 9.0 which is above the growth optimum for the organism (pH 6 to 8).

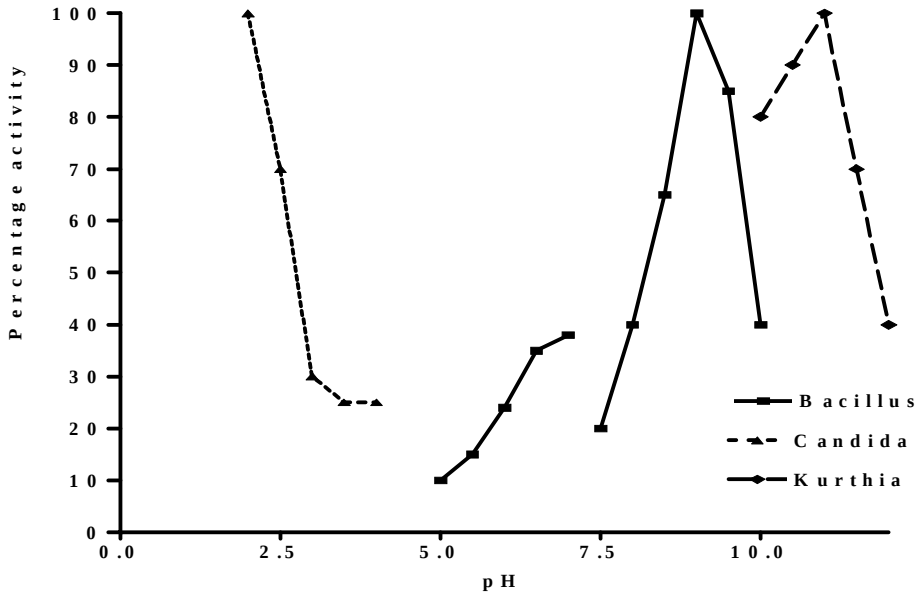

Figure 2. The effect of pH on esterase activity. In the range 2 to 4 acetate buffer was used, phosphate buffer from 5 to 7.5 and tris-HCl from 7.5 to 11. All buffer concentrations were 0.1M; activity was determined using flurorescein dibutyrate with a blank control at the same pH and buffer condition.

The enzymes were stable to a range of pH values from 5 to 10 for several hours.

Effect of Temperature.

The effect of temperature on esterase activity is shown in Fig. 3. The two esterases show very different temperature optima as would be expected from a mesophilic and a thermophilic organism. In the case of the mesophilic enzyme the growth temperature and the enzyme optimum are the same at 28 ^{0}, but in the case of *B. stearothermophilus* the growth optimum is 55 ^{0} , but the temperature optimum is 120^0.

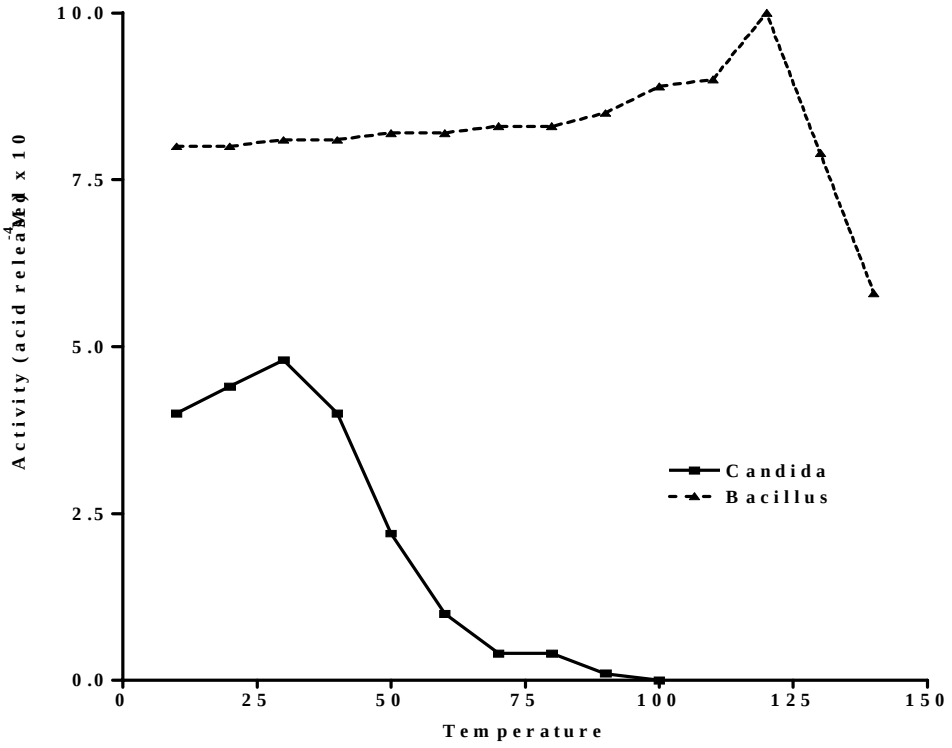

Figure 3. The effect of temperature on the activity of the esterases from *C. lipolytica* and *B. stearothermophilus*.

For the *Candida* esterase the optimum temperature was determined at pH 6.0 using a fluorimetric assay, the sample was equilibrated for one minute before activity

measurements were made. The optimum for the *Bacillus* enzyme was determined by hydrolysis of tributyrin in a sealed tube for two hours, followed by titration of the acid liberated, using a control without enzyme to correct for thermal hydrolysis at elevated temperatures.

The optimum temperature of 120^0 is exceptionally high,and compares with that of the extreme thermophile *Sulpholobus solfataricus* (18) where 5'methylthio-adenosine-phosphorylase had an optimum of 120^0 and *Pyrococcus furiosus* protease at 115^0.

The Arrhenius plot (Fig 4.) was discontinuous and concave upwards, a feature unusual in enzyme-catalysed reactions, although at temperatures above 120^0 rapid inactivation occurs .

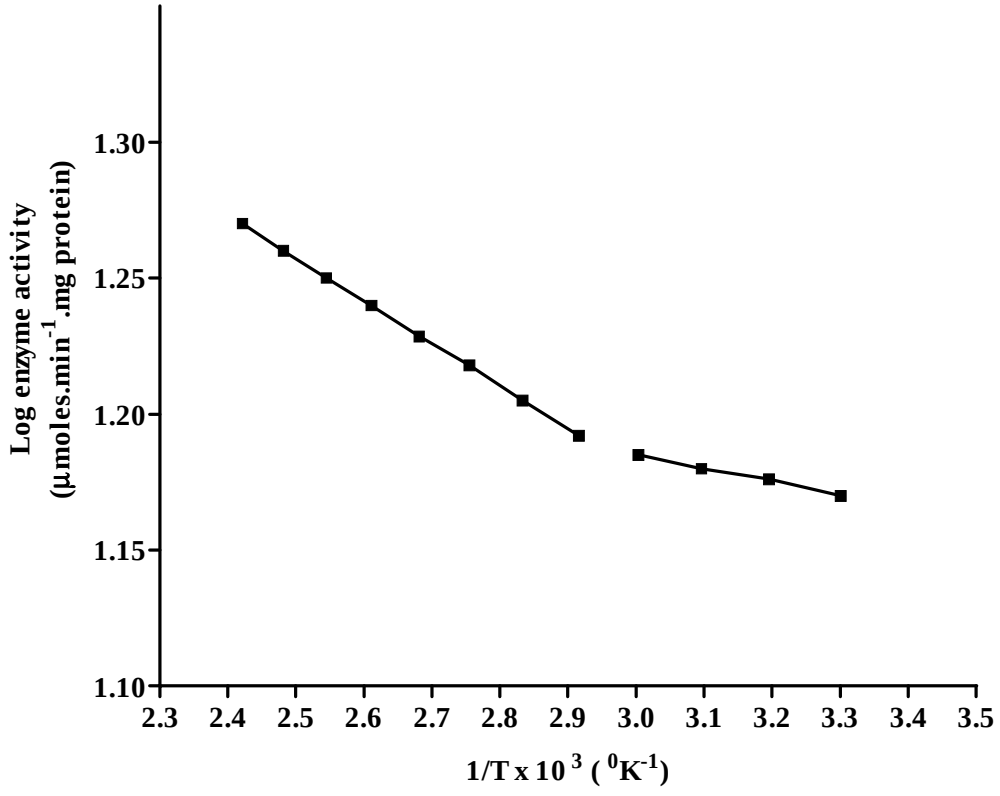

Figure 4. Arrhenius plot for *B. stearothermophilus* esterase.

Such plots are observed when a reaction system consists of two parallel reactions with different efficiencies, where the activation energies are such that one dominates at a higher temperature and the other at a lower temperature (19).

The energy of activation (E_a) from 30^0 to 70^0 was 355 cal $mole^{-1}$ and from 80^0 to 120^0 was 948 cal $mole^{-1}$.

The thermostability of these small esterases is high, although the thermophilic enzyme is more stable, as might be expected

Figure 5 shows the stability over a 100 hour period, with the *Candida* esterase having a half life of 2 hours at 50^0, very stable for a mesophilic organism, and the *Bacillus* esterase retained 95% of its activity at 70^0 at 100 hours. Even at 90^0 the enzyme had a half life of 12 hours.

Increased thermostability is the sort of property that might be useful in the extracellular environment. Robust enzymes which persist will maximise the substrate returns on the investment in extracellular proteins. From the economic viewpoint a small thermostable extracellular enzyme is efficient providing its activity is similar to that of large enzymes.

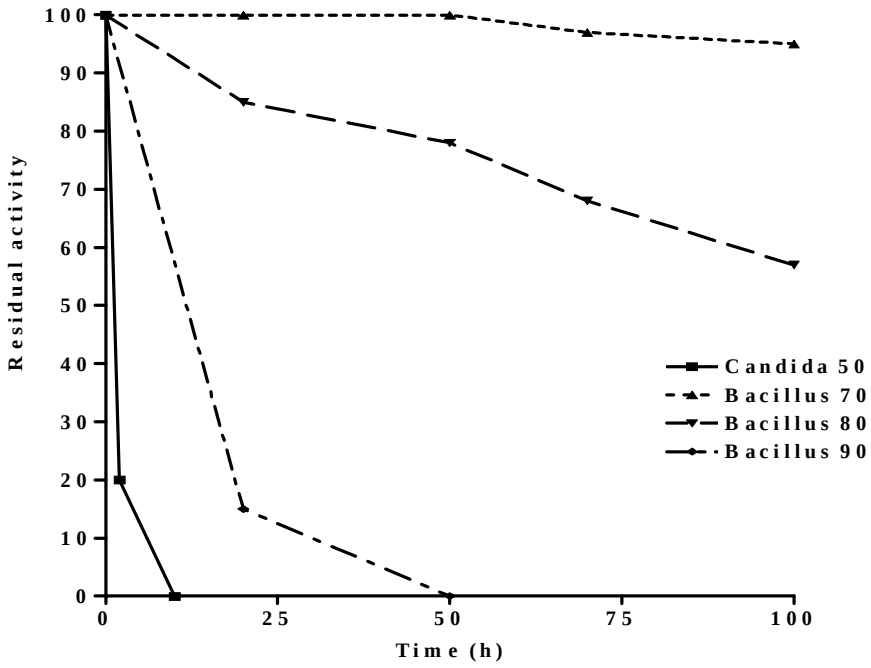

Figure 5 Thermostability of the esterases.

Specificity.

The two esterases differ both in their specificity towards triglycerides and towards the chain length of the fatty acid chains.

Figure 5 shows that the *Candida* esterase shows a preference for shorter chain lengths, with a peak at butyric although it is still effective against palmitic acid side chains with about 20% of the relative activity shown towards butyrate esters. This preference is seen both with fluorescein diesters and glycerol esters.

Products of the hydrolysis of triglycerides were separated by HPLC on a Lichrosorb Si60 column with a mass detector (Sedex 55) using a mobile phase of toluene-hexane (1:1) (solvent A) and toluene-ethyl acetate (3:1) plus 1.2% formic acid (solvent B). A gradient of 1 - 50% B over 10 minutes and 50 - 100% B from 10 to 15 minutes, with 100% B from 15 to 40 minutes was used.

The *Candida* esterase showed a peak of 2,3 diglyceride in the early stages of hydrolysis , with 1,3 diglyceride in small quantities appearing after all triglyceride was hydrolysed.

The *Bacillus* esterase on the other hand showed 1, 3 diglyceride only.

In both cases the diglyceride was slowly hydrolysed further to monoglyceride and butryric acid .

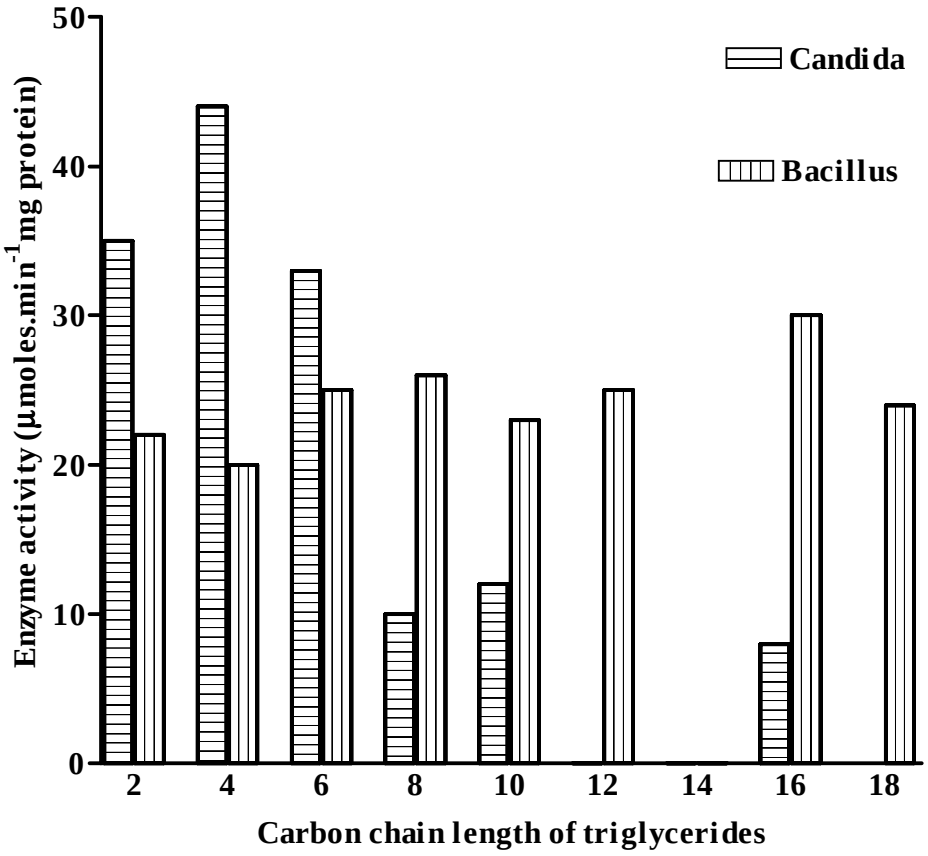

Figure 6 Esterase specificity

Conclusions.

It is apparent that the lower limit on the size of naturally occurring enzymes is well below 10 kDa, the *Bacillus* esterase described here is only 1.57 kDa and is little more than a peptide.

The range of activities so far seen is limited to simple hydrolysis, which is not unexpected given the small size; the scope for the binding of substrates with recognition between similar groups must be limited. Esterases, lipases and proteases have been described, but so far not carbohydrate or nucleic acid degrading activities.

Despite the limited substrate binding possibilities both esterases show specificity towards triglycerides, and these enzymes, with their considerable thermostability and reasonable activity, are of interest in industrial processes. Stability can be further enhanced by immobilisation which retains activity.

It is not known how widespread such enzymes are but preliminary screens show many thermophiles have enzyme activities in the micorenzyme size range.

It is noteworthy that the two esterases described appear to have different mechanisms of activity. The larger esterase from *C.lipolytica* has an ferric iron active site, where the metal catalyses an acid hydrolysis of the substrate. This is demonstrated by the pH dependence and the effect of dialysis.

The *Bacillus* esterase appears to use the metal ion to maintain the conformation of the peptide, but the pH optimum is not consistent with metal catalysis, nor is the effect of dialysis consistent with an active site metal ion, although the effect of chelators such as EDTA is consistent with the involvement of metal ions in an important functional role.

The active site sequences for a number of esterases show great similarity with the sequence Gly-Glu-Ser*-Ala-Gly being conserved and Glu/Asp -Ser being very common in the next two positions. The amino acid composition of the microenzyme from B stearothermophilus contains the seven amino acids found in the active site

sequence although the sequence is not known. The peptide is N terminal blocked, possibly by a formyl group. If this hypothesis is substantiated the mechanism whereby a confomrational change in such a small protein could cleave the ester bond will be of interest. The possible number of hydrogen bonds etc. is small but the role of metal ions in stabilisation may be significant.

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