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ORIGINAL PAPER

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Improved thermal stability and activity in the cold-adapted lipase B from *Candida antarctica* following chemical modification with oxidized polysaccharides

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Abstract In order to improve the thermal stability ($t_{1/2}$) and activity of lipase B from cold-adapted *Candida antarctica* (CALB), amino groups of the enzyme were chemically linked to a range of oxidized polysaccharides using a range of reducing agents. By chemically modifying CALB using 0.1% dextran (250 kDa) at pH 8.6 for 10 days using borane–pyridine complex as reducing agent, increased thermal stability ($t_{1/2}$, 168 min at 70°C) and activity (65% higher specific activity) was achieved compared to the unmodified enzyme ($t_{1/2}$, 18 min at 70°C). Improvements in thermostability were generally better with high molecular weight polymers such as dextran (40 and 250 kDa) or ficoll (70 and 400 kDa) in comparison to low molecular weight inulin (5 kDa). The shape of the polymer also appeared to be important with elongated, elipsoidal-shaped dextran providing better thermostabilization than spherical-shaped ficoll. Borane–pyridine complex was found to be a good, non-toxic reducing agent for improving thermostability, compared with sodium borohydride and sodium cyanoborohydride. An interesting finding was that, in all cases, specific activity of the modified enzymes increased with a concomitant increase in thermostability. This response defies the general principle of a trade-off between activity and stability, and demonstrates that chemical modification provides new avenues for improving the thermal stability of enzymes from psychrophiles without sacrificing their activity.

Keywords Enzyme thermal activity/stability · Psychrophile · Chemical modification · Lipase

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Abbreviations BPC: Borane–pyridine complex · CALB: *Candida antarctica* lipase B · DAP: Dialdehyde polysaccharide · SBH: Sodium borohydride · SCBH: Sodium cyanoborohydride

Introduction

Cold-adapted enzymes catalyse reactions effectively at low temperature (Gerday et al. 2000; Cavicchioli et al. 2002; Feller and Gerday 2003; Cavicchioli and Siddiqui 2004). As a result they have found application and have been evaluated for their potential in a range of industries (Cavicchioli et al. 2002; Cavicchioli and Siddiqui 2004). The inherently high specific activity of cold-adapted enzymes generally accompanies a low thermostability (Feller and Gerday 2003; Cavicchioli and Siddiqui 2004). An improved range of biotechnological opportunities would arise if cold-adapted enzymes could be thermally stabilized while maintaining, or improving their specific activity.

Triacylglycerol lipases (EC. 3.1.1.3) hydrolyze carboxylic esters into diacylglycerol and carboxylate anion, and are widely used in biotechnological processes to hydrolyze insoluble fats and oils and for synthesizing esters (Svendsen 2000; Benjamin and Pandey 1998). The psychrophilic yeast, *Candida antarctica*, expresses two lipases (A and B) with different physiochemical properties. Lipase B (CALB) is less thermostable, smaller in size, and more acidic than lipase A (Patkar et al. 1993). The CALB has been used for ester synthesis, desymmetrization and production of peracids in laboratory, pilot and commercial scale operations (Heldt-Hansen et al. 1988; Bjorkling et al. 1992; Zhang et al. 2003).

All lipases possess a serine-protease-like active-site triad, consisting of a Ser as the nucleophile, His as the basic residue, and Asp or Glu as the acidic residue (Uppenberg et al. 1994a, b). The CALB contains the sequence, Thr-x-Ser-x-Gly around the active-site Ser, whereas in all other microbial and mammalian lipases

Thr is replaced by Gly (Patkar et al. 1997). When Thr was replaced by a Gly in a CALB site-directed mutant (expected to create local flexibility), thermostability increased (rather than decreased) with a concomitant decrease in specific activity (Patkar et al. 1997). The unexpected increase in thermostability and the decrease in specific activity was thought to arise from the replacement of Thr side-chains with Gly enhancing contacts between secondary structural elements around the active-site (Patkar et al. 1997). Despite the availability of a crystal structure for CALB (Uppenberg et al. 1994a, b) it has been proven difficult to rationalize how to introduce single amino acid replacements in order to confer improvements in thermostability without compromising activity.

Recently, directed evolution (rather than site-directed mutagenesis) was used to improve the thermal stability and specific activity of CALB (Zhang et al. 2003). Directed evolution has been reported to be laborious and costly (Venkatesh and Sundaram 1998a, b), however, it does provide the means for selecting mutants with improved properties (Tao and Cornish 2002). The impressive improvements reported by Zhang et al. (2003) clearly demonstrate the value of this approach for modifying the properties of CALB.

To modify the properties of an enzyme, a complementary approach to recombinant DNA technology, is chemical modification. Benefits of chemical modification include affordability, accessibility to generally available laboratory infrastructure, speed in which improvements can be obtained, and applicability to native, recombinant, wild-type or mutant enzymes (Rashid and Siddiqui 1998; Siddiqui et al. 1999, 2000, 2004; Bokhari et al. 2002). By avoiding modification of the active-site and targeting enzyme surface groups, chemical modification can generate improvements in enzyme stability and efficiency (Mozhaev et al. 1998; Eijsink et al. 2004).

Naturally occurring and artificially produced glycoproteins are good examples of enzymes that have improved thermostability properties afforded by the carbohydrate moieties linked to their surface groups (Marshal 1978; Vankova et al. 1994). Glycoproteins can be synthesized by linking oxidized sugar polymers (dialdehyde polysaccharide, DAP) to enzyme amino groups in the presence of a reducing agent (Lenders and Crichton 1984; Rajalakshmi and Sundaram 1995; Venkatesh and Sundaram 1998a, b). Sodium borohydride ($NaBH_4$) (Means and Feeney 1968) or sodium cyanoborohydride ($NaCNBH_3$) (Friedman et al. 1974; Lenders and Crichton 1984; Venkatesh and Sundaram 1998a, b) have been used as reducing agents for this purpose. A disadvantage of the strong reducing agent, sodium borohydride (SBH), is that reductive amination also reduces the active aldehyde groups of DAP back to hydroxyl groups, thereby limiting the number of attachments DAP can make to the enzyme. In contrast, sodium cyanoborohydride (SCBH) is a very mild reducing agent which does not reduce aldehyde groups of DAP, and therefore provides the potential for forming multi-point attachments between polysaccharide and enzyme. The greatest drawback of SCBH is its toxicity caused by the cyanide. An alternative reducing agent to SBH and SCBH that has not previously been used for enhancing the thermostability of an enzyme is borane–pyridine complex (BPC). The BPC is a relatively inexpensive, non-toxic, and mild reducing agent (Pelter et al. 1984).

In this study we evaluated the use of SBH, SCBH, and BPC to link a variety of polymeric sugars to the surface amino groups of CALB. This chemical modification approach resulted in simultaneous improvements in the thermostability and specific activity of CALB. We found that BPC was an effective reducing agent, and identified trends in polysaccharide shape and length that correlated with the level of improvement in enzyme thermostability. The broad range of improvements that were generated highlighted the utility in this type of chemical modification for improving the activity and thermal stability of CALB. These findings, in combination with our previous studies on a cold-adapted shrimp alkaline phosphatase (Siddiqui et al. 2004), demonstrate the potential that chemical modification holds for modifying the properties of cold-adapted enzymes.

Materials and methods

Chemical modification of CALB by DAP

To generate oxidized polysaccharides (DAPs), aqueous solutions (1% w/v) of polysaccharides (dextran 40 and 250 kDa, ficoll 70 and 400 kDa, and inulin 5 kDa) were prepared. Sodium meta-periodate ($NaIO_4$) was added to a final concentration of 50 mM and incubated at room temperature in the dark for 2 h, followed by incubation for 24 h at 4°C in the dark (Rajalakshmi and Sundaram 1995; Venkatesh and Sundaram 1998a, b). The reaction was stopped by the addition of 500 µl of ethylene glycol. The oxidized polysaccharides were dialyzed using cellulosic dialysis membranes of appropriate cut-off values (5 and 1.2 kDa). All oxidized polysaccharides were stored in 1 ml aliquots at 4°C.

CALB (2.5 mg) was diluted in an appropriate phosphate (0.02 M Na_3PO_4/H_3PO_4, 0.15 M NaCl, pH 7.5 or 0.1 M K_2HPO_4/H_3PO_4, pH 7.13) or borate (50 mM boric acid/sodium borate, pH 8.6) buffer. The DAPs were added (0.05–0.7%) to the CALB solution (Lenders and Crichton 1984; Rajalakshmi and Sundaram 1995; Venkatesh and Sundaram 1998a, b) and the solutions were mixed on a carousel.

To couple DAPs to CALB, appropriate reducing agents were added. For SBH, a solution containing lipase and DAP was incubated at room temperature and stirred for 3–10 days. Solid SBH was added to a final concentration of 0.2 M (Means and Feeney 1968). After 1–2 h, all samples were dialyzed against 50 mM K_3PO_4/H_3PO_4, pH 7 buffer to remove reagents. Alternatively, the reaction was stopped by adding 500 µl of 25% gly-

cine to give a final concentration of 8% glycine, followed by dialysis. For SCBH, CALB solution, DAP and 35 mM SCBH were incubated with stirring at room temperature for 3–10 days in a buffer containing 15 mM Na_3PO_4/H_3PO_4 (pH 7.5) and150 mM NaCl (Lenders and Crichton 1984; Venkatesh and Sundaram 1998a, b). The reaction was stopped by the addition of 350 µl of 25% glycine solution and 600 µl of coupling buffer (50 mM $NaCNBH_3$ in 0.2 M NaCl and 20 mM Na_3PO_4, pH 7.5). After 4–5 h incubation at room temperature the modified lipase was dialyzed against 50 mM K_3PO_4/H_3PO_4, pH 7 buffer. For BPC, 60 mM (final concentration) was incubated at room temperature for 3–10 days with CALB solution and DAP (Pelter et al. 1984; Wong et al. 1984). The reaction was stopped by the addition of 25% glycine solution (final concentration of 8%), with subsequent dialysis using 50 mM K_3PO_4/H_3PO_4, pH 7. The final concentration of unmodified and modified CALB was 1.25 mg ml^{-1} .

Lipase activity determination

A solution (600 µl) containing 0.4% Triton X-100 and 0.1% gum Arabic in 50 mM K_3PO_4/H_3PO_4 (pH 7) was added to 70 µl of 0.375% emulsified *p*-nitrophenyl palmitate in iso-propanol. The mixture was equilibrated at 30°C, and 20–70 µl of lipase enzyme was added and incubated for 1–4 h at 30°C. When sufficient yellow color had formed (A410, ~0.6), the reaction was stopped by the addition of 670 µl of 2% Trizma base. The reaction mixture was centrifuged at 15,366 *g* for 30 min and the absorbance at 410 nm read against a reagent blank (Mayordomo et al. 2000).

The same quantity of lipase was used for all chemical modifications (see section above). After chemical modification all samples were dialyzed against buffer, and unmodified and modified samples were made up to the same volume. As a result, the same amount of modified and unmodified enzyme was used in all lipase assays. Protein concentration was determined by the Bradford assay (Kruger 2002). The specific activity was calculated as units mg^{-1} protein, where one unit of activity is defined as the amount of lipase that releases 1 µmol of *p*-nitrophenol per min under the assay conditions using the extinction coefficient of 15 mM^{-1} for *p*-nitrophenol.

Results and discussion

Chemically modifying CALB with DAPs (dextran 40 and 250 kDa, ficoll 70 and 400 kDa, and inulin 5 kDa) using reducing agents (SBH, SCBH, and BPC) led to a simultaneous increase in the stability and activity of the modified enzymes (Table 1). Stability (half-life of inactivation) increased by up to ninefold (dextran-250 modified-CALB using BPC as reducing agent at pH 8.6), and specific activity by up to 80% (inulin using BPC at pH 7.3). It was notable that specific activity was never reduced by chemical modification, despite the gains in thermal stability.

Modifier size and shape

Improvements in thermal stability were higher with high molecular weight polymers dextran (40 and 250 kDa) and ficoll (70 and 400 kDa) compared to low molecular weight (5 kDa) inulin (Table 1). For example, using the highest concentration of DAP incubated for 10 days with BPC as the reducing agent, the $t_{1/2}$ for dextran and ficoll modification ranged between 90 and 168 min, compared to 57 min for inulin. In contrast, the molecular weight of the individual dextran (40/250 kDa) or ficoll (70/400 kDa) sugars had little impact on the ability of either polymer to enhance stability (146/168 and 99/90 min, respectively) (Table 1). The improved thermal stability afforded by the larger modifiers may be caused by several factors. Multi-point attachments facilitated by the larger polymers may enhance surface rigidity and

Table 1 Stability and activity of modified and unmodified CALB

DAP	Unmodified[a]	Dextran							Ficoll				Inulin			
MW (kDa)		40				250			70		400		5			
Reducing agent		SBH	SCBH	BPC		SCBH	BPC		BPC		SCBH	BPC	SBH	SCBH	BPC	
DAP conc. (%)		0.15	0.12	0.37	0.10	0.12	0.37	0.10	0.37	0.10	0.12	0.37	0.10	0.30	0.24	0.74	0.20
Incubation time (day)		3	3	5	10	3	5	10	5	10	3	5	10	3	3	5	10
Incubation pH		8.60	7.50	7.13	7.1, 8.6	7.50	7.13	7.1, 8.6	7.1	7.1, 8.6	7.50	7.13	7.1, 8.6	8.60	7.50	7.13	7.1, 8.6
Half-life (min)																	
65°C (pH 7)	22 (16)[a]	77	86	–	–	63	–	–	–	–	80	–	–	26	70	–	–
70°C (pH 7)	18 (6)[a]	79	–	162	50, 146	–	124	56, 168	139	81, 99	–	114	56, 90	–	–	63	40, 57
Specific activity (%)	100	118	125	125	120, 158	125	175	150, 165	162	140, 168	132	172	155, 157	–	125	180	160, 160

SBH 200 mM sodium borohydride, *SCBH* 35 mM sodium cyanoborohydride, *BPC* 60 mM borane–pyridine complex, *Specific activity* percent lipase activity per mg protein relative to unmodified CALB, *DAP* dialdehyde polysaccharide (oxidized polymer)

[a] Values in *parentheses* for unmodified CALB in the presence of non-oxidized 40 kDa dextran

retard a conformational shift towards a denatured state of the protein. Additionally, the polyhydroxyl polymers, ficoll and dextran, are likely to be more favorably hydrated thereby strengthening hydrophobic interactions (Arakawa and Timasheff 1982). As a result they may more effectively decrease protein–water interactions creating a more stable protein. Stabilization by the larger polymers may also arise from a greater level of masking on the CALB surface. An analogous effect was attributed to the masking by benzophenone derivatives of non-polar clusters present on the surface of cold-adapted shrimp alkaline phosphatase (Siddiqui et al. 2004).

Dextran appeared to be better than ficoll for improving thermal stability (see data highlighted above from Table 1). Dextran is a predominantly linear (1–6 bonds) polymer with some branches (1–3 bonds) and exists as an elongated random coil in solution (Bohrer et al. 1979). Ficoll has extensive cross-linking between sucrose and epichlorohydrin molecules and is a rigid spherical structure in solution (Bohrer et al. 1979). It is possible that dextran can more effectively wrap around CALB, produce more linkages with CALB, and reduce the interaction of water molecules on the enzyme surface.

pH of chemical modification reaction

Employing a high pH (8.6), rather than low pH (7.1–7.5) during modification produced better improvements in thermal stability. This is illustrated by comparing pH 8.6–7.1 for CALB modification for 10 days in the presence of BPC (Table 1). The stability increased the most at high pH for dextran where a threefold difference was observed between pH 8.6 and 7.1. A higher pH will result in more amino-groups on the surface of CALB to be un-protonated, thereby increasing their reactivity towards aldehyde groups of the oxidized polymers. This is consistent with increasing thermal stability being mediated by increasing the number of linkages between enzyme and sugar polymer.

Reducing agent

The individual biochemical properties of the three reducing agents (BPC, SBH, and SCBH) dictated the chemical modification procedures that were used for modifying CALB (see Materials and methods). The mild reducing agents, BPC and SCBH, were added at the start of the incubation with the DAP to promote continuous conversion of imine groups into more stable amine groups, without destroying the reactive aldehyde groups of the DAP. In contrast, the strong reducing agent SBH could not be added at the start of the reaction because it reduces aldehyde groups into hydroxyl groups, thereby destroying the potential for bond formation with an amino group of the enzyme. Consistent with this, BPC and SCBH tended to provide better improvements than SBH (Table 1). For example, CALB modified with 0.15% dextran-40 at pH 8.6 had a $t_{1/2}$ of 79 and 146 min for SBH and BPC, respectively (Table 1). In view of the greatly reduced toxicity of BPC compared to SCBH (Lenders and Crichton 1984; Pelter et al. 1984; Wong et al. 1984; Venkatesh and Sundaram 1998a, b), our findings demonstrate an important potential that may be realized in biotechnology by adopting this reducing agent for chemical modification.

Improving thermal stability without compromising activity

A broad range of studies comparing homologous, low- and high-temperature adapted enzymes, and structure/function studies comparing mutant and wild-type enzymes have demonstrated that a trade-off exists between activity and stability in most enzymes (Gerday et al. 1997; Carrea and Colombo 2000; D'Amico et al. 2001; Roovers et al. 2001; Feller and Gerday 2003; Georlette et al. 2004). Site-directed mutagenesis studies of CALB have demonstrated this principle with a Thr to Gly mutant exhibiting increased thermostability and decreased specific activity (Patkar et al. 1997, 1998). In contrast, a directed evolution study generated a CALB mutant with ~20-fold increased stability and activity (Zhang et al. 2003). Aside from this study, there are few reports of mutations or modifications of cold-adapted enzymes that have produced enzymes, which defy the principle of activity-stability trade-off (Miyazaki et al. 2000; Siddiqui et al. 2004). A study that achieved this using chemical modification was that of Siddiqui et al. (2004), which reported the use of tetracarboxy-benzophenone derivatives to modify shrimp alkaline phosphatase. The data presented in this study demonstrate that chemical modification enhanced activity and stability towards *p*-nitrophenol palmitate. As lipases can hydrolyze a broad range of substrates, it will be interesting to determine whether the modifications affect the ability to hydrolyze other substrates, or whether the modification has also affected substrate specificity.

In combination with the present work, the findings from molecular-based and chemical-based modification procedures demonstrate an ability to generate modified, cold-adapted enzymes with unanticipated thermal activity/stability properties. This highlights the potential to improve the properties of other industrially important cold-adapted enzymes, and provides new opportunities for probing the structure/function relationships that govern enzyme activity and stability. A particularly interesting question that arises from our study is whether the activity and stability of the CALB mutant obtained from the directed evolution study (Zhang et al. 2003) can be further improved using our chemical modification approach.

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References

Arakawa T, Timasheff SN (1982) Stabilization of protein structure by sugars. Biochemistry 21:6536–6544

Benjamin S, Pandey A (1998) *Candida rugosa* lipases: molecular biology and versatility in biotechnology. Yeast 14:1069–1087

Bohrer MP, Deen WM, Robertson DR, Troy JL, Brenner BM (1979) Influence of molecular configuration on the passage of macromolecules across the glomerular capillary wall. J Gen Physiol 74:583–593

Bokhari SA, Afzal AJ, Rashid MH, Rajoka MI, Siddiqui KS (2002) Coupling of surface carboxyls of carboxymethylcellulase with aniline via chemical modification: extreme thermostabilization in aqueous and water-miscible organic mixtures. Biotechnol Prog 18:276–281

Carrea G, Colombo G (2000) Coupling high enzyme activity and stability. TIBTECH 18:401–402

Cavicchioli R, Siddiqui KS (2004) Cold-adapted enzymes (Chapter 32) In: Pandey A, Webb C, Soccol CR, Larroche C (eds) Enzyme technology. AsiaTech Publishers, New Delhi pp 608–631

Cavicchioli R, Siddiqui KS, Andrews D, Sowers KR (2002) Low-temperature extremophiles and their applications. Curr Opin Biotechnol 13:253–261

D'Amico S, Gerday C, Feller G (2001) Structural determinants of cold-adaptation and stability in a large protein. J Biol Chem 276:25791–25796

Eijsink VGH, Bjork A, Gaseidnes S, Sirevag R, Synstad B, Burg BV, Vriend G (2004) Rational engineering of enzyme stability. J Biotechnol 113:105–120

Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol 1:200–208

Fields PA, Somero GN (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A_4 orthologs of Antarctic notothenioid fishes. Proc Natl Acad Sci USA 95:11476–11481

Georlette D, Blaise V, Collins T, D'Amico S, Gratia E, Hoyoux A, Marx JC, Sonan G, Feller G, Gerday C (2004) Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev 28:25–42

Gerday C, Aittaleb M, Arpigny JL, Baise E, Chessa J-P, Garsoux G, Petrescu I, Feller G (1997) Psychrophilic enzymes: a thermodynamic challenge. Biochimica et Biophysica Acta 1342:119–131

Gerday C, Aittaleb M, Bentahir M, Chessa JP, Claverie P, Collins T, D'Amico S, Dumont J, Garsoux G, Georlette D, Hoyoux A, Lonhienne T, Meuwis MA, Feller G (2000) Cold-adapted enzymes: from fundamentals to biotechnology. TIBTECH 18:103–107

Heldt-Hansen HP, Ishi M, Patkar SA, Hansen TT, Eigtved P (1988) In: Whitaker JR, Sonnet PE (eds) Biocatalysis, agricultural biotechnology. ACS symposium series, vol 389, pp 158–172

Homann MJ, Vail R, Morgan B, Sabesan V, Levy C, Dodds DR, Zaks A (2001) Enzymatic hydrolysis of a prochiral 3-substituted glutarate ester; an intermediate in the synthesis of an NK_1/NK_2 dual antagonist. Adv Synth Catal 343:744–749

Koops BC, Papadimou E, Verheij HM, Slotboom AJ, Egmond MR (1999) Activity and stability of chemically modified *Candida antarctica* lipase B adsorbed on solid supports. Appl Microbiol Biotechnol 52:791–796

Kruger NJ (2002) The Bradford method for protein quantitation. In: Walker JM (ed) The protein protocols, handbook. Humana Press, New Jersey, pp 15–21

Lenders JP, Crichton RR (1984) Thermal stabilization of amylolytic enzymes by covalent coupling to soluble polysaccharides. Biotechnol Bioeng 26:1343–1351

Marshal JJ (1978) Manipulation of the properties of enzymes by covalent attachment of carbohydrate. Trends Biochem Sci 3:79–81

Mayordomo I, Gil-Randez F, Prieto JA (2000) Isolation, purification and characterization of a cold-active lipase from *Aspergillus nidulans*. J Agric Food Chem 48:105–109

Means GE, Feeney RE (1968) Reductive alkylation of amino groups in proteins. Biochemistry 7:2192–2201

Miyazaki K, Wintrode PL, Grayling RA, Rubingh DN, Arnold FH (2000) Directed evolution study of temperature adaptation in a psychrophilic enzyme. J Mol Biol 297:1015–1026

Mozhaev VV, Siksnis VA, Melik-Nubarov NS, Galkantaite NZ, Denis GJ, Butkus EP, Zaslavsky BY, Mestechkina NM, Martinek K (1998) Protein stabilization via hydrophilization. Covalent modification of trypsin and alpha-chymotrypsin. Eur J Biochem 173:147–154

Patkar SA, Bjorking F, Zundai M, Schulein M, Svendsen A, Heldt-Hansen HP, Gormsen E (1993) Purification of two lipases from *Candida antarctica* and their inhibition by various inhibitors. Ind J Chem 32B:76–80

Patkar SA, Svendsen A, Kirk O, Groth IG, Borch K (1997) Effect of mutation in non-consensus Thr-X-Ser-X-Gly of *Candida antarctica* lipase B on lipase specificity, specific activity and thermostabilty. J Mol Catal B Enzymatic 3:51–54

Pelter A, Rosser RM, Mills S (1984) Reductive aminations of ketones and aldehydes using borane–pyridine. J Chem Soc Perkin Trans 1:717–720

Rajalakshmi N, Sundaram PV (1995) Stability of native and covalently modified papain. Protein Eng 8:1039–1047

Rashid MH, Siddiqui KS (1998) Carboxyl group modification: high temperature activation of charge neutralized and charge reversed beta-glucosidase from *Aspergillus niger*. Biotechnol Appl Biochem 27:231–237

Roovers M, Sanchez R, Legrain C, Glansdorff N (2001) Experimental evolution of enzyme temperature activity profile: selection in vivo and characterization of low-temperature-adapted mutants of *Pyrococcus furiosus* ornithine carbamoyltransferase. J Bacteriol 183:1101–1105

Servi S (1992) Microbial reagents in organic synthesis. Nato Science Series: C, Springer, Heidelberg Berlin New York

Siddiqui KS, Shemsi AM, Anwar MA, Rashid MH, Rajoka MI (1999) Partial and complete alteration of surface charges of carboxymethylcellulase by chemical modification: thermostabilization in water–miscible organic solvent. Enzyme Microbiol Technol 24:599–608

Siddiqui KS, Najmus Saqib AA, Rashid MH, Rajoka MI (2000) Carboxyl group modification significantly altered the kinetic properties of purified carboxymethylcellulase from *Aspergillus niger*. Enzyme Microbiol Technol 27:467–474

Siddiqui KS, Poljak A, Cavicchioli R (2004) Improved activity and stability of alkaline phosphatases from psychrophilic and mesophilic organisms by chemically modifying aliphatic or amino groups using tetracarboxy-benzophenone derivatives. Cell Mol Biol 50:657–667

Svendsen A (2000) Lipase protein engineering. Biochim Biophys Acta 1543:223–238

Tao H, Cornish VW (2002) Milestones in directed evolution. Curr Opin Chem Biol 6:858–864

Uppenberg J, Hansen MT, Patkar S, Jones TA (1994a) The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. Structure 2:293–308

Uppenberg J, Patkar S, Bergfors T, Jones TA (1994b) Crystallization and preliminary X-ray studies of lipase B from *Candida antarctica*. J Mol Biol 235:790–792

Vankova H, Pospisilova M, Ticha M, Turkova J (1994) Stabilization of trypsin by glycosylation. Biotechnol Tech 8:375–380

Venkatesh R, Sundaram PV (1998a) Upward shift of thermotolerance of cold water fish and mammalian trypsins upon chemical modification. Ann NY Acad Sci 864:512–516

Venkatesh R, Sundaram PV (1998b) Modulation of stability properties of bovine trypsin after in vitro structural changes with a variety of chemical modifiers. Protein Eng 11:691–698

Wong DWS, Osuga DT, Feeney RE (1984) Pyridine–borane as reducing agent for proteins. Anal Biochem 139:58–67

Zhang N, Suen W-C, Windsor W, Xiao L, Madison V, Zaks A (2003) Improving tolerance of *Candida Antarctica* lipase B towards irreversible thermal inactivation through directed evolution. Prot Eng 16:599–605