Chemically stabilized subtilisins in peptide synthesis

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Abstract

We have stabilized alcalase^{TM} and subtilisin Carlsberg (SC) against heat by chemical modification with ethylene glycol *bis*-succinimidyl succinate (EGNHS), a procedure not previously reported for subtilisins. The increases in thermal stability at 65°C were 1.8-fold and 4.7-fold respectively. Caseinolytic activity of alcalase in aqueous buffer was unchanged following modification but apparent K_m of SC decreased 2.5-fold. Native and modified forms of both enzymes synthesized the tripeptide Z-Tyr-Gly-Gly-NH_2 under kinetic control in mixtures based on 0.2M barbitone buffer, pH 9.0 and 50% v/v dimethyl formamide/ barbitone buffer. Native enzymes gave faster rates of product formation than their modified counterparts in buffer but differences were much less pronounced in the mixed solvent. We also compared native alcalase and SC in terms of thermal stability, tolerance of organic solvents and autolysis. Alcalase was approx. 4.6-fold more stable than SC at 65°C and was more tolerant of acetone, acetonitrile

and 1,4-dioxane. Alcalase underwent autolysis at approx. half the rate of SC. Against succinyl-Ala-Ala-Pro-Phe substrates, alcalase showed a much higher esterase/ amidase ratio (567) than SC (29) in aqueous buffer but this was reversed in 50% v/v dimethylformamide, where the esterase/ amidase ratios were 43 and 113 respectively.

Keywords: Alcalase, subtilisin Carlsberg, stabilization, chemical modification, peptide synthesis.

Abbreviations: ACN, acetonitrile; AOT, aerosol OT (dioctyl sodium sulfosuccinate); BCA, bicinchoninic acid; C_{50}, co-solvent concentration (% v/v) where 50% of aqueous enzymatic activity remains; CC, cyanuric chloride; CLEC, cross-linked enzyme crystal; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EGNHS, ethylene glycol-*bis*(succinic acid N-hydroxysuccinimide ester); *k*, first-order inactivation rate constant; log P, $\log_{10}$ of a solvent's partition coefficient in a 1-octanol/water two-phase system; NPC, nitrophenol carbonate; NTCI, N-trans-cinnamoylimidazole; OSP, oxidized sugar polymer; PEG, polyethylene glycol; sAAPF-pNA, 3-Carboxy-propionyl-$(Ala)_2$-Pro-Phe-p-nitroanilide; sAAPF-SBzl, 3-Carboxy-propionyl-$(Ala)_2$-Pro-Phe-thiobenzylester; SBL, subtilisin *Bacillus lentus*; SC, subtilisin Carlsberg; SDS, sodium dodecyl sulphate; T_{50}, temperature where 50% of room-temperature enzymatic activity remains after 10 min; T_m, unfolding temperature (where a polypeptide is 50% unfolded); $t_{½}$, half-life (0.693/*k*, where *k* is the first-order inactivation constant); THF, tetrahydrofuran; Z, benzyloxycarbonyl.

Introduction

Bacterial subtilisins are versatile serine proteases that are much used in industry (Gupta et al., 2002). Common variants include subtilisin BPN' from *B. amyloliquefaciens*, subtilisin Carlsberg from *B. licheniformis* and Savinase and Esperase (both registered trade marks) from the highly alkalophilic *B. lentus* (Von der Osten et al., 1993) Alcalase (a commercial protease from Novozymes) is best known as a detergent protease but also finds use in the synthesis of peptides (Hou et al., 2006a,b, 2005), chiral derivatives of succinic acid (Bailey et al., 1999) and optically active amino acids and peptides (Chen & Wang, 1999). Unfortunately, however, these and other proteases are prone to autolysis, thermoinactivation and denaturation in non-aqueous solvents. Strategies to reduce these adverse effects (and to enhance the enzyme's catalytic properties and applicability for a particular purpose) include immobilization (see Table 1), protein engineering and chemical modification.

Protein Engineering Different subtilisins have been subjected to extensive protein engineering by a variety of mutational strategies, including DNA shuffling (Bryan, 2000; Gupta et al., 2002; Ness et al., 1999; Wells & Estell, 1988). Mutational targets within the protein have included catalytic amino acids, substrate binding regions and substitutions aimed at stabilizing the molecule, as reviewed by Bryan (2000) and Gupta (2002). More recently, Strausberg et al. (2005) coevolved high catalytic activity and hyperstability in calcium-free subtilisin (i.e. from which calcium binding site A had been deleted) by randomizing 12 amino acid positions in succession. Both stability and catalytic properties were used to choose the best amino acid at each site; each such mutant became the parent clone for the next round of mutagenesis. When combined, the 12 individual mutations hugely increased the enzyme's half-life at 75°C. Stabilizing mutations had

no direct influence on catalytic properties and only substitutions at or near the substrate binding surface influenced the catalytic constants. Detailed analysis revealed insights into the evolution of substrate binding, acylation and the rate of product release.

Replacement of a 16-amino acid sequence in mesophilic subtilisin E by a thermophilic, more hydrophobic, model sequence (DiTursi et al. 2006) increased the protein's melting temperature by 13°C and gave a greatly extended half-life at 60°C. The stabilizing replacement segment (GSELDVMAPGVSIQST in single-letter amino acid code; positions 193-208) was based on a strand-turn-strand motif consensus sequence identified by bioinformatic algorithms using Bayesian methodology.

Using an involved five-stage procedure, Ruan et al. (2004) constructed a switchable subtilisin BPN' that can act as a useful tool in single-step protein purifications. This enzyme is immobilized in a HiTrap NHS Sepharose column and acts as an affinity ligand for the tight binding of fusion proteins tagged with the subtilisin prodomain. Once non-binding contaminants have been washed through the column, addition of fluoride triggers catalytic cleavage of the target protein-prodomain link. The target protein can be isolated and the column regenerated. Ten different fusion proteins have been purified by this method.

Meta-analysis of reports of engineered subtilisins indicates that substitutions yielding improvements are statistically biased towards positions showing significant phylogenetic variation and towards orthologous replacements (Cochran et al., 2006)

A notable mutant form, subtiligase, has been engineered especially for peptide synthesis. Jackson et al. (1994) produced milligram quantities of fully active ribonuclease A using 'subtiligase' to ligate six peptide ester fragments in high yield. This enzyme is derived from subtilisin BPN' via two mutations in the active site, Ser221 → Cys and Pro225 → Ala, which greatly increase its performance as a peptide ligase. Other uses, and improvements, of this novel catalyst are described by Chang et al. (1994), Braisted et al. (1997) and Atwell and Wells (1999).

Genetic manipulation-chemical modification (GM-CM) Some time earlier, Grøn and co-workers (1990) combined site-specific mutagenesis with chemical modification to achieve an oxidation-resistant form of subtilisin. Wild type subtilisin lacks cysteines: thus, a Cys substituted into recombinant subtilisin by site-directed mutagenesis can become a specific target for subsequent chemical modification with a thiol-specific reagent. The enzyme in question came from *Bacillus lentus* and was termed subtilisin 309. Here, Met 222 (prone to oxidation and situated beside the catalytic Ser 221) was replaced by Cys but the Cys was then thiomethylated by reaction with a thioalkylating reagent, methyl methane thiosulfonate (Me-MTS) to yield a thiomethylated Cys derivative, dubbed [Me-S-Cys^{222}] subtilisin. This procedure markedly increased the enzyme's resistance to hydrogen peroxide with minimal loss in performance. A similar strategy led to improved oxidative tolerance in the detergent protease, Maxacal™, an oxidation-resistant variant of which has been commercially marketed as Maxapem™ (Misset, 1993; van Eekelen et al., 1989).

Besides stability, a combined chemical modification-genetic manipulation (CM-GM) strategy has also been used to alter subtilisin's catalytic properties. Location of a single Cys at different positions in the protein chain followed by reaction with one of a range of alkyl methanethiosulfonates can alter specificity (DeSantis et al., 1998), esterase/amidase ratio

(Plettner et al., 1999) or pH-activity profile (DeSantis & Jones, 1998). Berglund et al. (1997) prepared two cysteine mutants of subtilisin *Bacillus lentus* (SBL) by substituting positions Asn62 and Leu217, located respectively in the S2 and S1' pockets. Each mutant was then reacted with different methanethiosulfonate compounds to yield a range of chemically modified mutants. Depending on the sulfonate, k_{cat}/K_m values against the tetrapeptide substrate sAAPF-pNA fluctuated, with peak vales up to 3.2- and 1.6-fold greater than wild type SBL for the Asn62 and Leu217 mutants respectively. Nednoor et al. (2004) covalently attached a fullerene derivative to the C-terminus of a mutant subtilisin. This facilitated immobilization of the enzyme on surfaces.

Chemical modification Chemical modification alone can stabilize subtilisin and influence its catalytic properties. Nakatsuka et al. (1987) found that the semisynthetic thiolsubtilisin, where the active site Ser221 is chemically converted to its thiol counterpart cysteine, functioned well in the coupling of short peptide segments with little secondary hydrolysis. Later, Wu and Hilvert (1989) chemically changed the active site Ser221 of subtilisin Carlsberg to selenocysteine. The resulting selenosubtilisin displayed notably higher aminolysis/hydrolysis ratios than either subtilisin Carlsberg or thiolsubtilisin. The procedure changed a proteinase into an acyl transferase sufficiently selective for use as a peptide ligase. Methylation of the ε2-N of the active site His of subtilisin BPN' (using methyl 2-hydroxy-2-phenylethanesulfonate) eliminated much of the amidase activity but sufficient esterase activity remained to facilitate peptide synthesis reactions via aminolysis (Zhong et al., 1991).

Cross-linked enzyme crystals (CLECs) are very stable and versatile biocatalysts. Microcrystalliine preparations of a given enzyme are prepared and crosslinked with

glutaraldehyde. Since they form a re-usable solid, CLECs can be considered to be immobilized enzymes. CLECs of subtilisin have been described; these were active and very stable in both aqueous and organic media and could perform peptide synthesis, transesterifications and enantio- and regio-selective reactions (Wang et al., 1997; Haring et al., 1999). Optimal stabilization of subtilisin Carlsberg occurred with modification of three lysines (53% recovery of initial activity) while modification of more lysines resulted in further loss of activity (down to 25%; Tüchsen & Ottesen, 1977). These crosslinked subtilisin crystals showed increased thermostability and decreased autolysis and remained active in dry acetonitrile (Tüchsen & Ottesen, 1977) Too many crosslinks may lead to activity losses and/or to precipitation (Margolin, 1996). It is also possible to convert subtilisin CLEC to seleno-subtilisin CLEC and so change the protease to a peroxidase (Haring & Schreier, 1999).

Covalent coupling of oxidized sucrose polymers (OSPs) of different sizes (400 and 70 kDa) and of polyglutaraldehyde to subtilisin Carlsberg increased the enzyme's kinetic, thermal and thermodynamic stabilities. Stability gains were greatest for 400 kDa OSP, where T_m increased by 8.4°C and the half-life at 60°C increased six-fold in presence of 10 mM Ca^{2+}. In the absence of Ca^{2+}, resistance to thermoinactivation was also enhanced, but to a lesser degree. Half-life enhancements for OSP70 and PGA were 5.2- and 2.9-fold respectively. The various modifying agents showed a similar rank order of effectiveness in increasing the protein's unfolding temperature (T_m) and resistance to denaturant-induced two-state unfolding. Of the nine lysines in the enzyme polypeptide, three, two and four were modified by OSP400, OSP70 and polyglutaraldehyde respectively (Srimathi et al., 2006).

Plou and Ballesteros (1994) covalently attached various long chain fatty acids to subtilisin Carlsberg. The modified enzyme Octanoyl-subtilisin showed a $t_{½}$ of 2.9 hours which was ~2-fold higher than the native enzyme at 65°C. Palmitoyl-subtilisin had a ~3-fold increase in its $t_{½}$ value compared to the native.

Kwon et al. (1999) modified subtilisin Carlsberg with PEG or aerosol-OT (AOT) and found that both modified forms had higher transesterification activity than native in organic solvents. The protein conformation of modified subtilisins Carlsberg underwent notable changes in water-miscible organic solvents but not in water-immiscible ones; the α-helix content declined notably in DMSO.

Detergents containing proteases are not suitable for use with natural protein fibres such as silk and wool. In an attempt to retain subtilisin's stain removal abilities in detergents while avoiding damage to protein fibres, PEG was covalently coupled to the enzyme. Attachment of poly ethylene glycol (PEG) to subtilisin via a 1,1'-carbonyldiimidazole activator of PEG yielded 90% recovery of enzyme activity compared with only 50% recovery when cyanuric chloride was used instead as the activating compound. PEG-modified subtilisins caused 95% less damage to wool fibres but retained good stain removal abilities and had half-lives nearly twice as long as native enzymes (Schroeder et al., 2006).

Chemical modification with the bifunctional crosslinker ethylene glycol *bis*-succinimidyl succinate (EGNHS) has been previously shown to enhance trypsin's resistance to denaturing influences such as high temperatures (Murphy & Ó'Fágáin, 1998). Here, we describe the effects

of EGNHS treatment on the stabilities and peptide synthesis abilities of the classical subtilisin Carlsberg and of the commerical variant known as Alcalase™.

Materials and Methods

Alcalase® was a gift from Novozymes, 2880 Bagsvaerd, Denmark. Fisher Chemicals supplied solvents (except DMSO) and NaCl while BDH/Merck supplied KCl, sodium barbitone, orthophosphate salts and Tween®20. Bicinchoninic acid (BCA) protein assay kit (Smith et al., 1985) was from Pierce (Rockford, IL 61105, USA). 3-Carboxy-propionyl-$(Ala)_2$-Pro-Phe-benzyl thioester (sAAPF-SBzl), Z-Tyr-OMe and H-Tyr-Gly-Gly-NH_2 were from Bachem. Sigma-Aldrich supplied subtilisin Carlsberg, casein, 3-carboxy-propionyl-$(Ala)_2$-Pro-Phe-p-nitroanilide (sAAPF-pNA), N-trans-cinnamoylimidazole (NTCI), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), ethylene glycol-*bis*(succinic acid N-hydroxysuccinimide ester) (EGNHS), sodium tetraborate, Gly-Gly-NH_2.HCl, tris-(hydroxymethyl) aminomethane (Tris), dimethylsulphoxide (DMSO) and all other chemicals.

Activity assay (Plettner et al., 1998).

Substrate solution (0.16mM sucAAPF-pNa in 0.1M Tris-HCl/20mM $CaCl_2$ pH 8.0, 300µl) was pipetted into microplate wells, followed by enzyme solution (0.01mg/ml enzyme in 0.1M NaH_2PO_4 adjusted to pH 8.4, 25µl). Reaction was allowed to proceed for 1 min and A_{405} read on a Labsystems Multiskan MS microplate reader. An enzyme-free blank was also included.

Autolysis assay was based on Bickerstaff and Zhou (1993). Enzyme solution (0.1mg/ml in 0.2M NaH_2PO_4, adjusted to pH 7.0) was prepared. Aliquots (5µl) of this solution were transferred to

wells of a microtitre plate followed by addition of Bradford reagent (250µl; Bradford, 1976). The plate was mixed gently and the absorbance read at 620nm (against blank) every hour up to 5 h.

Temperature profile and thermoinactivation Enzyme solution (0.01mg/ml in 0.1M borate/0.1M $CaCl_2$ adjusted to pH 9.0 with 5M HCl) was prepared together with a blank containing borate buffer only. Aliquots of enzyme solution (70µl) were heated to temperatures between 30-90°C (in 5°C increments) for 10 min, cooled on ice, returned to room temperature and assayed for residual amidase activity. In the case of thermoinactivation of enzyme solution at a constant 75°C or 65°C, 50µl aliquots were removed at intervals, cooled on ice, returned to room temperature and assayed for residual amidase activity.

Organotolerance Enzyme solution (0.1mg.ml^{-1}) in 0.1M KH_2PO_4 adjusted to pH 8.4/ organic solvent mixture in the range 0-90% (v/v) solvent was incubated at 30°C for 1 h. Residual amidase activity was then measured. Solvents used were acetone, acetonitrile (ACN), 1,4-dioxan, DMF, DMSO and THF.

Active site titration was after Zhong et al. (1991) and Bender et al. (1966). Solutions of NTCI (1mg/ml in ACN) and enzyme (1mg/ml in 3mM KH_2PO_4, 0.1M KCl, adjusted to pH8.2) were prepared. To a cuvette was added 975µl 0.1M Tris-HCl/0.1M NaCl pH 7.0. NTCI solution (20µl) was added and A_{335} measured (Unicam UV2 spectrophotometer) after 1 min. Enzyme solution (35µl) was added to the cuvette and the change in absorbance recorded after 5 s. A control containing NTCI alone was also measured. Readings were taken in triplicate.

Kinetic Studies (after Plettner et al., 1998)

Amidase Activity sAAPF-pNa solution (0.5mM) was prepared in 0.1M Tris-HCl/20mM $CaCl_2$, pH 8.0. Enzyme solution (50µl; 0.001mg/ml in 0.1M NaH_2PO_4, adjusted to pH8.4) was pipetted into rows A-G of a microtitre plate, with 50µl 0.1M NaH_2PO_4, adjusted to pH 8.4, in row H (the "loading plate"). A second microtitre plate (the "assay plate") was set up with 300µl sAAPF-pNa in each well. Enzyme solution (25µl) was transferred from the loading plate to the corresponding wells of the assay plate using a multi-channel pipette. Absorbance values were read every 30 s over 5 min at 405 nm on a Labsystems Multiskan MS microplate reader. Data were converted to rates in $M^{-1}s^{-1}$ using ε_{414} = 8581 $M^{-1}cm^{-1}$ for p-nitroaniline and the results of the active site titrations (above).

Esterase activity sAAPF-SBzl solution (1mM in 0.1M NaH_2PO_4 containing 0.375mM DTNB, adjusted to pH 7.2) was prepared and 25µl aliquots were dispensed into each well of the assay plate followed by 180µl NaH_2PO_4 solution, adjusted to pH 8.4. Enzyme solution (40µl; 0.001mg/ml in 0.1M NaH_2PO_4, adjusted to pH 8.4) was dispensed into rows A-G of the loading plate, with 0.1M NaH_2PO_4, adjusted to pH 8.4, in row H as the blank. Enzyme solution (25µl) was transferred from the loading to the assay plate as above. Absorbances were read every 5 s over 1 min at 405 nm on the Multiskan MS reader. Data were converted to rates in M s^{-1} using ε_{414} = 8708 $M^{-1}cm^{-1}$ for the thiophenolate ion and the results of the active site titrations (above).

Chemical modification with EGNHS Enzyme (5mg) was dissolved in 2.375ml 3mM KH_2PO_4, 0.1M KCl, adjusted to pH 8.2. EGNHS (5mg) was dissolved in 125µl DMSO and added slowly, with stirring, to the enzyme solution. Stirring continued for 20 min at room temperature (Murphy

and Ó'Fágáin, 1998) and reaction was then terminated by centrifugal gel filtration (Helmerhost and Stokes, 1980) on Sephadex™G-25 in an ALC Multispeed Centrifuge PK121.

pH Profile of caseinolytic activity Casein (2.5g) was dissolved in 100ml 2mM Tris solution by heating to 40°C with stirring. The cooled solution was adjusted to the required pH (varying from pH 6.3 to 10.8) using 1M HCl. A 1mg/ml enzyme stock solution was prepared in 3mM KH_2PO_4/0.1M KCl adjusted to pH 8.2. The reaction mixture comprised 4ml casein substrate solution, 2ml 0.1M KCl, 2ml distilled water and 200µl enzyme stock solution. Proteolytic activity was monitored using a pH-stat (Metrohm 718 pH-STAT titrino). CO_2 was removed by bubbling nitrogen through the solution.

Casein digestion at pH 8.0 Casein was dissolved as above, allowed to cool, adjusted to pH 8.0 (using 5M HCl) and serial dilutions of this substrate solution were prepared. A 1mg/ml enzyme stock solution was prepared in 3mM KH_2PO_4/0.1M KCl adjusted to pH 8.0. The reaction mixture comprised 4ml casein solution, 2ml 0.1M KCl and 2ml distilled water. Enzyme solution (100µl) was then added to start the reaction and activity was monitored using the pH-stat. CO_2 was removed by bubbling nitrogen through the solution.

Synthesis of tripeptide Tyr-Gly-Gly-NH_2 This method was developed from Klein et al. (2000) and Chen et al. (1991). 0.2mM Gly-Gly-NH_2 was prepared in 0.2M sodium barbitone, pH 9.5. 0.1M Z-Tyr-O-Me was prepared in DMF and equilibrated at -15°C for 10 min. Enzyme solution (1mg/ml) was prepared in 3mM KH_2PO_4, 0.1M KCl adjusted to pH 8.2. Z-Tyr-O-Me solution (250µl) was mixed with Gly-Gly-NH_2 (245µl) and 5M aqueous triethylamine (5µl). Enzyme

solution (5µl) was added to this mixture and the solution incubated at 4°C. At intervals, 50µl aliquots were removed, added to 375µl of 50% (v/v) aqueous acetonitrile and analysed on a Varian HPLC system with a Prostar 410 autosampler, Star 9050 UV/Vis detector and a Hypersil C18 column (250mm x 4.6mm); flow rate 1ml min^{-1}, detection at 280nm, mobile phase 50% (v/v) aqueous acetonitrile. Samples (50µl) were injected onto the column using the autosampler. Substrates and products were compared with their corresponding standards. The effects of enzyme concentration (1-2 mg/ml) and reaction temperature were investigated similarly.

Results

Native subtilisins: stability

Autolysis Of the two enzymes, Subtilisin Carlsberg was more prone to autolysis: it lost 40% amidase activity after 3 h while alcalase lost only 20% activity under the same conditions (data not shown).

Thermal stability Subtilisin Carlsberg and alcalase had T_{50} values of 68°C and 72°C respectively at pH 9.0 (10 min incubations; data not shown). At a constant 75°C, thermoinactivation data for both enzymes obtained over the first 10 min fitted well to a first order exponential decay (Enzfitter program; Biosoft, Cambridge, UK) but deviated at longer times; alcalase showed a slightly higher $t_{½}$ value (5 min, $k = 0.139 \pm 0.012$) than subtilisin Carlsberg (4 min, $k = 0.17 \pm 0.02$). Both enzymes had completely lost activity after 20 min.

Organo-tolerance assay Table 2 shows that ACN had the most detrimental effect on subtilisin Carlsberg, with 40% v/v ACN leading to a 50% loss of amidase activity. Alcalase, in contrast, retained 50% amidase activity up to 72% v/v ACN. Alcalase also retained 100% amidase activity up to 90% v/v 1,4-dioxan but was vulnerable to DMSO: it lost 50% amidase activity at 48% v/v

DMSO, similar to subtilisin Carlsberg. Both enzymes retained 50% amidase activity up to DMF values slightly exceeding 50% v/v (Table 2).

Native subtilisins: catalysis

Active site titration The NTCI procedure indicated that native subtilisin Carlsberg was 73% active while native alcalase was 78% active; these figures were used in calculations of k_{cat}/K_m values (below). Bender et al. (1966) found that a typical sample of crystalline subtilisin Carlsberg contained 57% (w/w) active enzyme and concluded that the inert material arose mainly from autolysis.

Amidase and Esterase Michaelis-Menten Kinetics For subtilisin Carlsberg, optimal amidase conditions were found to be 0.6 nM enzyme and 0.5 mM sAAPF-pNA over 540 s, while optimal esterase conditions were 1.1 nM enzyme and 11 μM sAAPF-SBzl over 95 s. Optimum concentrations for alcalase were 3 nM (amidase) and 0.8 nM in the esterase assay; other conditions were as for subtilisin Carlsberg. Table 3 shows k_{cat}/K_m values obtained for both enzymes in aqueous buffer and in 50% DMF. Subtilisin Carlsberg had an overall esterase/amidase ratio of 28 in aqueous buffer. This compares well with the esterase/amidase ratio of 44 obtained in similar experiments with subtilisin *Bacillus lentus* (SBL; Plettner et al., 1998). Amidase activity of subtilisin Carlsberg is 3.7-fold less in 50% DMF than in aqueous buffer; in contrast, the esterase values correspond closely. This situation is reversed in the case of alcalase: here, amidase values correspond closely but esterase activity is 10-fold less in the mixed solvent. Nevertheless, the esterase/amidase ratio of alcalase in aqueous solution (567) is much greater than that of subtilisin Carlsberg (Table 3).

EGNHS-modified subtilisins

Active site titration with NTCI indicated that the EGNHS-modified forms of both enzymes contained approximately 36-37% active material (compared with 73-78% for native; see above), so there was some loss of catalytic activity during the course of the chemical modification procedure. This coincided with lesser protein contents in both cases: alcalase retained 50% of its pre-modification protein concentration while subtilisin Carlsberg retained 62.5%.

Thermal stability At 65°C, EGNHS-modified alcalase was the most stable enzyme form (Fig. 1): 50% of initial amidase activity remained at 100 min, compared with a 56 min half-inactivation period for native alcalase (1.8-fold stabilization). Chemical modification stabilized subtilisin Carlsberg to a greater degree (4.7-fold): EGNHS-Carlsberg lost 50% activity in 56 min, compared with only 12 min for native. Data obtained at this lower temperature (65°C) did not fit a first-order rate equation, so true half-lives could not be calculated.

Casein digestion Caseinolytic activities of native and modified forms of both subtilisin Carlsberg and alcalase followed Michaelis-Menten kinetics and apparent V_{max} and K_m values are shown in Table 4 (Enzfitter programme: Biosoft, Cambridge, UK). EGNHS-Carlsberg hydrolysed casein ~3-fold more efficiently than the native. In contrast, native and EGNHS-alcalase showed similar caseinolytic activities.

Synthesis of Z-Tyr-Gly-Gly-NH_2 by native and EGNHS-modified subtilisins

Enzyme concentration Molar concentrations of products were determined from a calibration curve constructed using stock solutions of commercially sourced standards.

Reaction mixtures containing subtilisin Carlsberg and alcalase were incubated at 4°C. Both 1mg enzyme/ml and 2mg enzyme/ml gave the same yield of tripeptide product; the lower concentration was used subsequently to conserve enzyme.

Effect of temperature Reaction solutions were incubated at 37°C (Fig 2A), 25°C (Fig 2B) and 4°C (Fig 2C). The greatest yield occurred with native subtilisin Carlsberg at 25°C, where 1.6 mmol of tripeptide product was synthesised. Yield was slightly lower (1.4 mmol) at 37°C, where alcalase was the more efficient enzyme. The lowest yield was at 4°C, where only 1 mmol of Z-Tyr-Gly-Gly-NH_2 was formed by native subtilisin Carlsberg. At all temperatures tested, the modified enzymes gave lower tripeptide yields in aqueous solution than did the native forms. At 4°C, both modified alcalase and modified subtilisin Carlsberg synthesised only 0.8 mmol of tripeptide product.

Effect of DMF Modified subtilisin Carlsberg yielded the most tripeptide (1.12mmol product over 60 min) in 50% v/v DMF solution (Fig 2D). Modified subtilisin Carlsberg had been less active than native in the aqueous solution at the same temperature (Fig 2C).

Unexpectedly, given its decreased esterase/amidase ratio in the DMF mixture (Table 3), native alcalase also showed slightly enhanced synthesis when 50% v/v DMF/ buffer was used in the synthesis mixture in place of the aqueous buffer component. The esterase/amidase ratio for alcalase in 50% v/v DMF was 13-fold lower than in aqueous solution, which would suggest that

alcalase would not be as efficient a catalyst for peptide synthesis in a mixed solvent. This is not borne out by the peptide synthesis results, however.

Discussion

We have compared subtilisin Carlsberg and commercial alcalase in terms of their thermal and solvent stabilities, their amidase, esterase and caseinolytic activities and their peptide synthesis abilities. We have also examined both enzymes' chemically stabilized counterparts following modification with the homobifunctional reagent EGNHS, which compound has previously been shown to stabilize trypsin and horseradish peroxidase (Murphy & Ó'Fágáin, 1998; O'Brien et al., 2001 respectively). Most published literature to date describes the stabilization of subtilisin by site-directed mutagenesis (e.g. Bryan, 2000) rather than by chemical modifications. Examples of the latter strategy have been outlined in the Introduction above. The present EGNHS chemical modification is both cheaper and easier to perform than site-directed mutagenesis and gives a notable stability gain, at least for subtilisin Carlsberg.

Stability of native subtilisins Native forms of both enzymes remain fully stable up to 60°C, as found by Hirata et al. (2003) for subtilisin Carlsberg. Subtilisin-type proteases possess 2 calcium binding site(s) and bound Ca^{2+} contributes to stability (Briedigkeit and Frömmel, 1989). Calcium ions were included in the pH 9.0 borate buffer used for thermal stability comparisons. In these, subtilisin Carlsberg steadily lost activity at 65°C and was completely inactive after 25 min (Fig. 1). In contrast, alcalase was still fully active at 25 min and retained 50% activity at 56 min.

Subtilisins catalyse the hydrolysis of proteins in aqueous solution but organic or mixed solvent systems are often required to effect enzymatic peptide synthesis reactions. Accordingly, we carried out organo-tolerance studies were to see how much activity the enzymes would retain in organic solvent-aqueous mixtures. Table 2 shows C_{50} values for subtilisin Carlsberg and alcalase in aqueous mixtures of various organic solvents. Acetonitrile, DMF and DMSO had the greatest effects on subtilisin Carlsberg (C_{50} <60 in each case); these are the solvents with the lowest log P values of those tested (Khmelnitsky et al., 1991). The lower the log P value, the greater the disruption of the essential bound water phase surrounding the enzyme (Nurok et al., 1999). Sears et al. (1999) found that freeze-dried subtilisin BPN' resuspended in DMF/water mixtures ranging 30-70% v/v DMF was quite stable, presumably due to decreased autolysis. Alcalase, in contrast, had a C_{50} value of 72 in acetonitrile compared to 40 for subtilisin Carlsberg. Both enzymes retained most activity in 1,4-Dioxan (Table 2); this was unexpected, as its log P value is low. Chen et al. (1991) found that alcalase had lost 50% activity after 15 min in 40% dioxane, a result that contrasts sharply with ours. Their experiment took place at 35°C with stirring and used 0.1 ml of alcalase in 25 ml of cosolvent mixture; pH was not specified. These conditions are quite different from our static experiment (see Methods). Sears et al. (1999) noted that vigorous stirring badly affected the stability of freeze-dried subtilisin BPN'.

Khmelnitsky et al. (1988, 1991) noted that the inactivation of an enzyme in certain water-miscible organic solvents is due to the solvent's absorbing water and stripping the enzyme of its hydration shell, which is essential for catalytic activity. It is thought that the lower the log P value, the greater distortion the solvent will cause to the enzymes' bound water (Nurok et al., 1999). However, some enzymes retain their bound water so tightly that a solvent molecule cannot replace the water molecules. Fitzpatrick et al. (1993) demonstrated that subtilisin Carlsberg

retained the same three-dimensional crystal structure in anhydrous acetonitrile as it did in water: 99 of 119 structural water molecules were not displaced from the crystal, even in anhydrous acetonitrile. The protein structure of the acyl-enzyme intermediate was virtually identical, although the locations of solvent molecules bound in the active sites of the acyl- and free enzymes differ between water and acetonitrile (Schmitke et al., 1998). However, aqueous mixtures of other hydrophilic organic solvents can cause detrimental effects to the enzyme, as they can strip away the water molecules bound to the enzyme and cause a decrease in catalytic activity (Gorman and Dordick, 1992).

Stability of EGNHS-modified subtilisins Both subtilisin Carlsberg and alcalase were reacted with the bifunctional crosslinker, EGNHS, which has a span of 16 Å and targets the lysine residues of the enzyme. Subtilisin Carlsberg contains nine lysine residues (SwissProt no. P00780) while subtilisin BPN' (subtilisin Novo) has 10 (SwissProt no. P00782), so there are numerous reactive sites on each protein.

Fig. 1 shows EGNHS-alcalase to be very stable: it retained 100% amidase activity for up to 85 min at 65°C, whereas native subtilisin Carlsberg had lost all activity after just 25 min at this temperature. EGNHS-alcalase had approximately a 2-fold increase in its apparent half-life versus native alcalase (estimated by inspection of Fig. 1: a true half-life could not be calculated since the activity loss was not first order.) EGNHS-subtilisin Carlsberg demonstrated a greater stabilization effect: it retained 50% activity up to 56 min, a ~4-fold increase over native in its apparent half-life at 65°C. This stability increase took place from a lower base, however, such that the thermal stability of EGNHS-Carlsberg closely resembled that of untreated alcalase. It is likely that the stabilizing effect of treatment with the bifunctional EGNHS has arisen from the formation of an

intramolecular crosslink (although this remains to be proven by, for example, comparison of tryptic fragments of native and modified subtilisins).

Using a purified preparation, Srimathi et al. (2006) recorded half-life increases of up to 9-fold following attachment of OSP400 to 2 of subtilisin Carlsberg's 9 lysines. Unfortunately, since their assay conditions differ from ours in terms of protein concentration, inactivation temperature and buffer pH, it is not possible to make direct comparisons with the stabilization effectiveness of the EGNHS modification. Yang et al. (1996) stabilized subtilisin Carlsberg by the attachment of PEG 5,000. Conjugation of PEG, previously activated by either cyanuric chloride or nitrophenol carbonate, to subtilisin took place in 0.1 M borate buffer, pH 9.0. Recoveries of starting activity were 88% and 93-95% for for the cyanuric chloride-activated (CC-PEG) and the nitrophenol carbonate-activated PEG (NPC-PEG) respectively. Amidase activity measurements indicated that both PEG-conjugated fractions were much more tolerant of heat (range 30°-60°C) and pH (range 6.5-9.5, 30°C). At 30°C, the NPC-PEG enzyme was much more stable than its CC-PEG counterpart; both were considerably more stable than the native, with half-lives ranging from 3 to 28 times greater than that of native subtilisin (according to the temperature). NPC-PEG derivative had a longer half-life than the CC-PEG subtilisin at all pH values investigated. Experiments suggested that the improved stability characteristics arose from decreased autolysis and not from increased molecular stability. Half-lives of all three subtilisins decreased as pH increased; both activity and autolysis of subtilisin increase with pH. PEG-modified subtilisins can be incorporated into polymers such as polyacrylates so as to achieve further stability gains (Yang et al., 1995). While PEG attachment seems to give greater recovery of pre-treatment activity and a greater stability enhancement than does our modification with EGNHS, since Yang et al. (1996)

used lower inactivation temperatures and slightly different methods, direct comparison with our results is not possible.

Catalysis by native and EGNHS-modified subtilisins Casein is the most frequently used substrate for protease activity under neutral and alkaline conditions. Beutel et al. (2002) tested seven different proteases, including alcalase and subtilisin, against casein substrates. Alcalase was the most efficient protease, hydrolysing the casein from 2mg/ml to 0.9mg/ml in two minutes; over the same period, subtilisin Carlsberg decreased the casein only from 2mg/ml to 1.9mg/ml. This agrees with the present results, where alcalase hydrolysed casein approximately twice as efficiently as subtilisin Carlsberg (Table 4). EGNHS-subtilisin Carlsberg showed a 2.7-fold greater proteolytic activity than did native (Table 4). We used both alcalase and subtilisin Carlsberg as supplied, without any further purification. Ferreira et al. (2003) observed numerous protein bands on an electrophoretogram of alcalase; the 27.3 kDa band corresponding to subtilisin Carlsberg comprised <20% of the overall protein content. Svendsen and Breddam (1992) have shown that alcalase preparations contain at least one other enzymatically active component, a glutamic acid-specific serine endopeptidase, besides the main alkaline protease activity. This may explain the increased caseinolytic efficiency of alcalase over subtilisin Carlsberg.

Kinetic results were determined for native subtilisin Carlsberg and alcalase using tetrapeptide substrates in both an aqueous and 50% (v/v) aqueous/DMF solvent system. Subtilisin Carlsberg's esterase/amidase ratio of 114 in presence of DMF (Table 3) represents a 4-fold increase over the corresponding aqueous value. The esterase k_{cat}/K_m value is scarcely altered but the amidase activity is noticeably less in the 50% (v/v) DMF/aqueous solution (Table 3), leading to a 4-fold

rise in the esterase/amidase ratio. This should increase its effectiveness in peptide synthesis, as aminolysis would be favored over hydrolysis. (Sewald & Jakubke, 2002).

Alcalase in aqueous solution showed the greatest esterase/amidase ratio (Table 3). It is used in washing detergent formulations and so is designed to operate at a moderate alkalinity in an aqueous environment. The present results indicate that alcalase would be the more suitable enzyme for peptide synthesis in an aqueous system. Alcalase showed an esterase/amidase ratio of 43 in 50% DMF, 13-fold less than the value in aqueous buffer. Here, it is the amidase activity that shows little change while there is a 10-fold decrease in esterase activity (Table 3). Since neither of the tetrapeptide substrates contain glutamic acid, it is extremely unlikely that any possible contaminating glutamic acid-specific endopeptidase (Svendsen & Breddam, 1992) was in any way an interfering factor.

Khmelnitsky et al. (1988) stated that maximal activity in organic solvent is achieved if the pH value of the starting aqueous enzyme solution corresponds to the pH optimum of its catalytic activity in water. The aqueous solution that was used in the kinetic studies was at pH 8.4 for both subtilisin Carlsberg and alcalase, which is in the optimum pH range for both enzymes. We did measure possible effects of solvent addition on pH of the reaction mixture but the concentrated 0.2 M buffer used in the aqueous solutions likely maintained the desired pH value.

Peptide synthesis by native and EGNHS-modified subtilisins Klein et al. (2000) synthesized the tetrapeptide Z-Val-Trp-Gly-Gly, using subtilisin Carlsberg to couple Z-Val-Trp-OMe with various nucleophilic components. Subtilisin Carlsberg showed a strong preference for Gly in the P_1' position and the nucleophile Gly-Gly-NH_2 gave the maximum product yield (90%);

accordingly, this was chosen as the nucleophile in the present experiments. In contrast, Chen et al. (1991) obtained the product Z-Tyr-Gly-Gly-OH using alcalase. We combined both these methods to synthesize the tripeptide Z-Tyr-Gly-Gly-NH_2 from the acyl donor Z-Tyr-OMe and the nucleophile Gly-Gly-NH_2 in a DMF/ Na barbitone system and compared the synthetic efficiencies of native and chemically modified subtilisin Carlsberg and alcalase.

We chose DMF as co-solvent for our peptide synthesis reactions because it is frequently used for this purpose and elsewhere in organic chemistry. Since the C_{50} values for subtilisin Carlsberg and alcalase in DMF were 56% and 58% v/v respectively, appreciable enzyme activity should remain at the 50% v/v DMF level. Klein et al. (2000) had to use 50% (v/v) DMF in order to dissolve Z-Tyr-OMe. It is essential to optimise the solvent system(s) used in enzyme-catalyzed peptide synthesis, as the solvents affect the enzyme stability, solubility of the substrate and the yield of the peptide product (Zhou et al., 2003).

Enzymatic peptide synthesis was performed under kinetic control, as this requires lower enzyme amounts and shorter reaction times than the alternative, thermodynamically-controlled, strategy. Synthesis of a peptide by a serine protease increases with the esterase/amidase ratio, as the acyl-enzyme intermediate is more likely to undergo aminolysis (leading to peptide synthesis) rather than hydrolysis in situations where the esterase/amidase ratio is high (Sewald & Jakubke, 2002; Plettner et al., 1999).

We performed peptide synthesis reaction at 4°C, 25°C and 37°C. After five minutes, the greatest yield of peptide was obtained at 4°C using native subtilisin Carlsberg: 0.9mmol compared to 0.7mmol for the same enzyme solution at 37°C. Lower reaction temperature can give higher

yields of peptide product (Jönsson et al., 1996). However, as time progressed, the optimum yields for both native and modified forms of alcalase and subtilisin Carlsberg were obtained at 25°C (Figure 2B), the temperature chosen by Klein et al. (2000).

Different researchers have chosen solvents other than DMF to effect subtilisin-mediated peptide synthesis. Using alcalase, Hou et al. (2005) synthesized benzoyl-Arg-Gly-Asp(NH_2)-OH by coupling benzoyl arginyl ethyl ester to Gly-Asp-$(NH_2)_2$. A tripeptide yield of 73.6% resulted from optimal conditions of ethanol/ Tris-HCl pH 8.0 (85:15 v/v) at 35°C for 8 h. Alcalase satisfactorily used the Arg derivative as acyl donor, demonstrating the broad specificity of the subtilisins but also reflecting the preference shown by subtilisin Carlsberg for Gly in the P_1' position (Klein et al., 2000). Hou et al. also successfully used alcalase to synthesize benzoyl Arg-Gly-NH_2 from Bz-Arg-O-Et and glycinamide (2006a; acetonitrile/ carbonate-bicarbonate buffer pH 10.0 (85:15 v/v), 45°C, 1 h, 82.9% yield) and Z-Asp-Ser-NH_2 (2006b; acetonitrile/ carbonate-bicarbonate buffer pH 10.0 (85:15 v/v), 35°C, 6 h, 75.5% yield). Getun et al. (2001) reported efficient formation of tetrapeptides (80-90% yields) of general formula Z-Ala-Ala-P_1-P_1'-*p*NA by SDS-subtilisin 72 hydrophobic ion-pair complexes in ethanol. The enzyme could accept as acyl donors N-protected tripeptides having unprotected basic or acidic residues in the P_1 position.

Subtilisin Carlsberg showed a 4-fold increase in esterase/amidase ratio when used in 50% (v/v) DMF compared with purely aqueous solution (Table 3). Alcalase, in contrast, unexpectedly had a decreased esterase/amidase ratio in 50% v/v DMF, so we decided to investigate the effect on the rate of Z-Tyr-Gly-Gly-NH_2 synthesis of using 50% v/v DMF/ buffer in place of the aqueous buffer component of the synthesis reaction mixture. Yield of the tripeptide product up to 20 min using EGNHS-subtilisin Carlsberg was about 50% greater in 50% v/v DMF compared with

aqueous buffer (contrast panels C and D of Fig. 2). Both forms of alcalase showed enhanced yields in the mixed solvent, with the greater improvement (~50%) for EGNHS-alcalase. EGNHS-alcalase and native subtilisin Carlsberg displayed equivalent peptide synthesis abilities in the two solvent systems. Since none of our enzymatic peptide synthesis reactions involve glutamic acid, any possible contamination with the glutamic acid-specific endopeptidase that is known to occur in alcalase preparations (Svendsen & Breddam, 1992) is unlikely to have influenced our alcalase-catalysed syntheses.

Conclusion

Alcalase shows slightly greater thermal stability than subtilisin Carlsberg. Alcalase is also more tolerant of the water-miscible solvents acetonitrile and 1,4-dioxan, and has a notably greater esterase/amidase ratio against tetrapeptide substrates in aqueous buffer, than subtilisin Carlsberg. Both enzymes can be thermally stabilized by chemical modification with EGNHS in a procedure that is easy to perform, is effective on samples that have not been extensively purified and is relatively cheap compared with the costs of mutagenesis. Native and EGNHS-modified forms of both enzymes successfully synthesised the tripeptide Z-Tyr-Gly-Gly-NH_2 with yields of up to 1.6 mmol; optimum conditions for both were at 25°C in aqueous buffer. Since these EGNHS-modified enzymes demonstrated enhanced stability and good activity, they could prove useful in the synthesis of other peptides and in further biocatalytic applications.

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Table 1. Some Recent Reports of Immobilized Subtilisins

Solid Particle	Type of Interaction	Subtilisin	Remarks	Ref.
Silica gel particles	Adsorption	Carlsberg	Used circular dichroism with rotating cell holder to monitor conformational changes in immobilized enzyme that has undergone inactivation in organic medium	(a)
Superparamagnetic carriers	Affinity adsorption: epoxy groups on carriers react with phenylboronic acid or benzamidine; these affinity ligands then bind enzyme	Carlsberg	Fe_3O_4 nanoparticles were coated by spraying suspension polymerization. Maximum adsorption capacity approx. 65 mg/g at pH 9.5. Both phenylboronic acid and benzamidine are inhibitors of subtilisin. Enzyme can be recovered by desorption under mild conditions	(b)
Poly(vinyl alcohol) cryogel	Covalent attachment: aldehyde-, epoxy- and vinylsulfone-containing solid phases prepared	Subtilisin 72	Improved enzyme stability in water and in mixtures with low water content. Hydrophilic support gel matrix retains internal water even in organic solvents	(c)
Impact-resistant alginate granules	Encapsulation	Subtilisin	Optimum enzyme activity obtained with granules formulated with 3% alginate, 10% starch, 10% TiO_2, 3% enzyme. Affords dust-free particles that	(d)

			are potentially useful in laundry detergents	
Protein-coated microcrystals	Water-soluble micron-sized crystalline particles are coated with biocatalyst. Preparation described in ref. (f).	Carlsberg	For use in organic solvents. Operational stability is solvent-dependent. Enzyme activity increases when coated onto solid-state buffers	(e,f)
Polystyrene beads surface-grafted with polyethylene glycol (PS-sg-PEG)	Covalent attachment: cyanuric Cl is coupled to terminal –OH groups of beads; the cyanuric Cl then reacts with free –NH_2 groups on enzyme	Subtilisin	Loading of up to 450-500 mg enzyme/ g beads achieved	(g)
Silica supports with different pore sizes and two types of chemical groups	Covalent attachment: glutaraldehyde-activated terminal –NH_2 groups, or carbonyl diimidazole-activated –OH groups, on the solid react with free –NH_2 groups on enzyme	Alcalase 2T	Higher total and specific activities, and 7-fold longer half-life at 50°C, obtained with particles having free –NH_2 groups. Immobilization was less effective, however, than with –OH-bearing particles. Larger pore sizes gave more effective immobilization	(h)
Crosslinked enzyme	Enzyme precipitated at pH 5.6, crosslinked with	Carlsberg	Examined enzyme activity and enantioselectivity in transesterification	(i)

crystals	1.5% glutaraldehyde		of *N*-acetyl-L-phenylalanine ethyl ester with *n*-propanol in different solvents	
-SH-bearing agarose or derivatized silica beads	Covalent, via –S-S- link to thiol-bearing supports	BPN’	Mutated Ser 145 or Ser 249 to Cys to enable site-specific immobilization on solid supports.	(j)

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Table 2: C_{50} values (% v/v) of various organic solvents for subtilisin Carlsberg and alcalase.

Solvent:	S. Carlsberg	Alcalase
THF	60	64
Acetone	60	70
1,4 – Dioxan	68	*
Acetonitrile	40	72
DMF	56	58
DMSO	48	48

C_{50} - Concentration of solvent leading to 50% loss of catalytic activity. * - Alcalase retained >50% activity up to 90% v/v 1,4 – Dioxan. Each assay was performed in triplicate. Most standard deviations were 5% or less; none exceeded 10%.

Table 3: Catalytic efficiency of native subtilisin Carlsberg and alcalase in aqueous (pH 8.0) and 50% v/v aqueous/DMF solution and the optimum values for enzyme and substrate concentration in these assays.

		[E] in well (nM)	[S] in well (μM)	k_{cat}/K_m ($s^{-1}M^{-1}$, $*10^{-5}$)	Esterase/ amidase
Subtilisin Carlsberg (aqueous)	Amidase	0.6	460	8.1	28
	Esterase	1.2	12	230	
Subtilisin Carlsberg (50% v/v DMF soln)	Amidase	2.7	460	2.2	114
	Esterase	1.3	12	250	
Alcalase (aqueous)	Amidase	3.1	460	1.1	567
	Esterase	0.8	12	610	
Alcalase (50% v/v DMF soln)	Amidase	2.8	460	1.4	43
	Esterase	1.3	12	61	

All plots of absorbance (405 nm) v. time had $r^2 \geq 0.99$ over the period of assay for both amidase and esterase activity. Each assay was performed in triplicate. Standard deviations were < 10%.

Table 4: Casein Digestion

	V_{max} (s^{-1}) ± 10%	K_m (mM) ± 30%	V_{max}/K_m ($s^{-1}mM^{-1}$)
Alcalase	31.3	0.07	447
EGNHS-alcalase	38.9	0.09	432
Subtilisin Carlsberg	37.3	0.15	246
EGNHS-Carlsberg	39.3	0.06	655

Assays were performed in duplicate.

Figure legends

Figure 1: Thermoinactivation at 65°C of (▲) native and (Δ) EGNHS-modified subtilisin Carlsberg and of (•) native and (o) EGNHS-modified alcalase in 0.1 M borate buffer/0.1 M $CaCl_2$ pH 9.0.

Figure 2: Synthesis of Z-Tyr-Gly-Gly-NH_2. ▲, subtilisin Carlsberg; Δ, EGNHS-modified subtilisin Carlsberg; ●, alcalase; ○, EGNHS-modified alcalase. Panel A, 37°C; Panel B, 25°C; Panel C, 4°C; Panel D, 4°C using 50% v/v DMF/ 0.2M Na barbitone pH 9.0 mixture in place of the barbitone buffer component.

Fig. 1

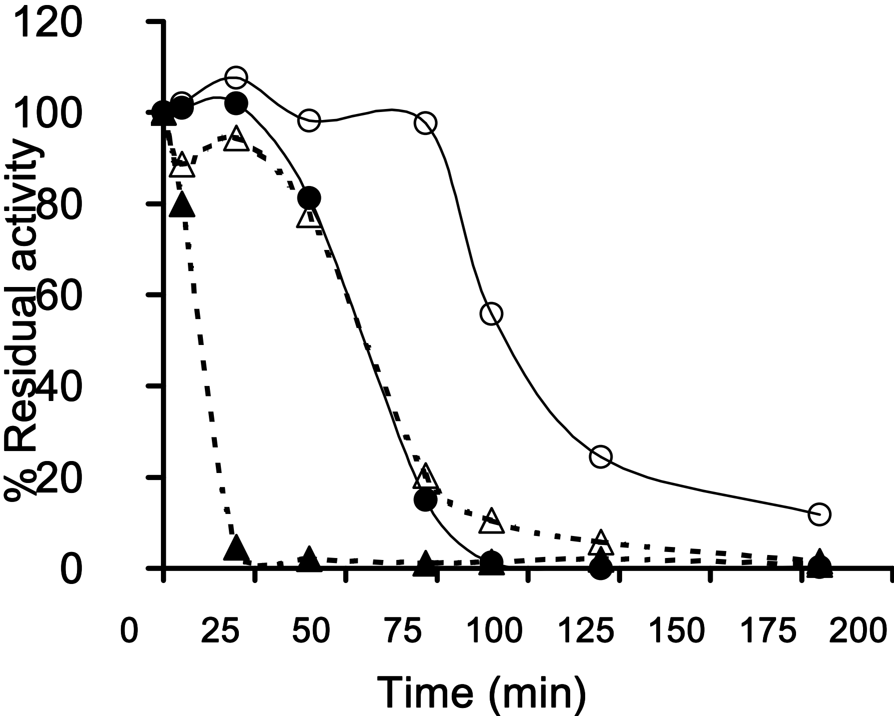

Fig.2

A

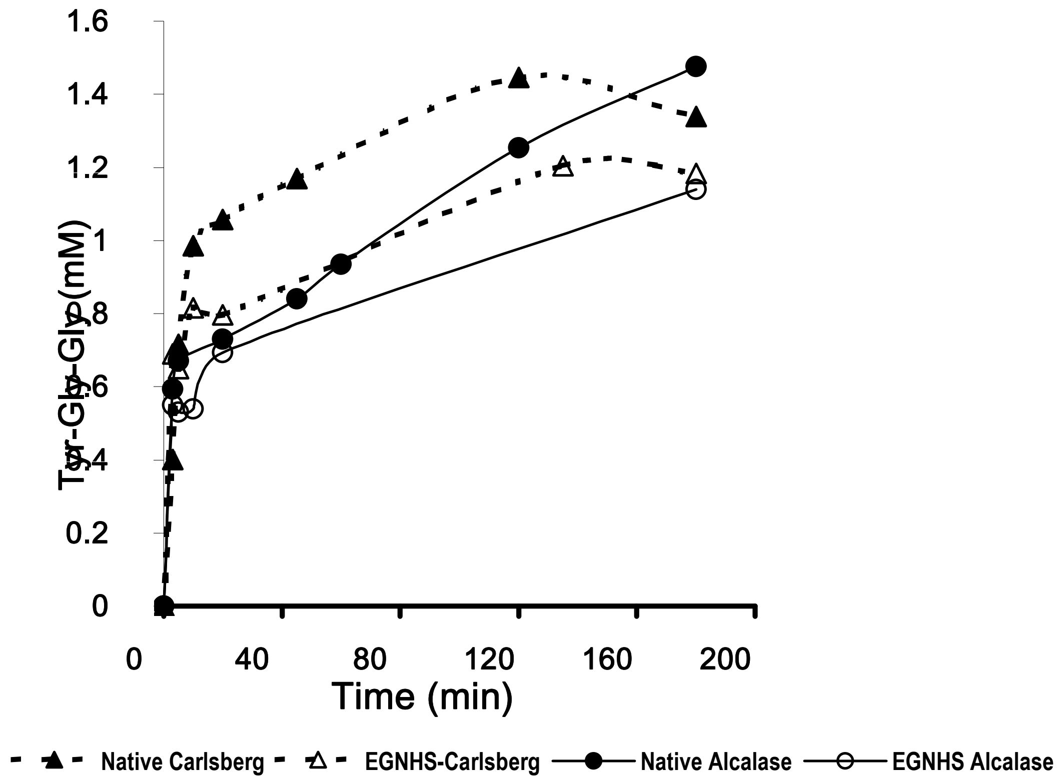

Fig. 2

B

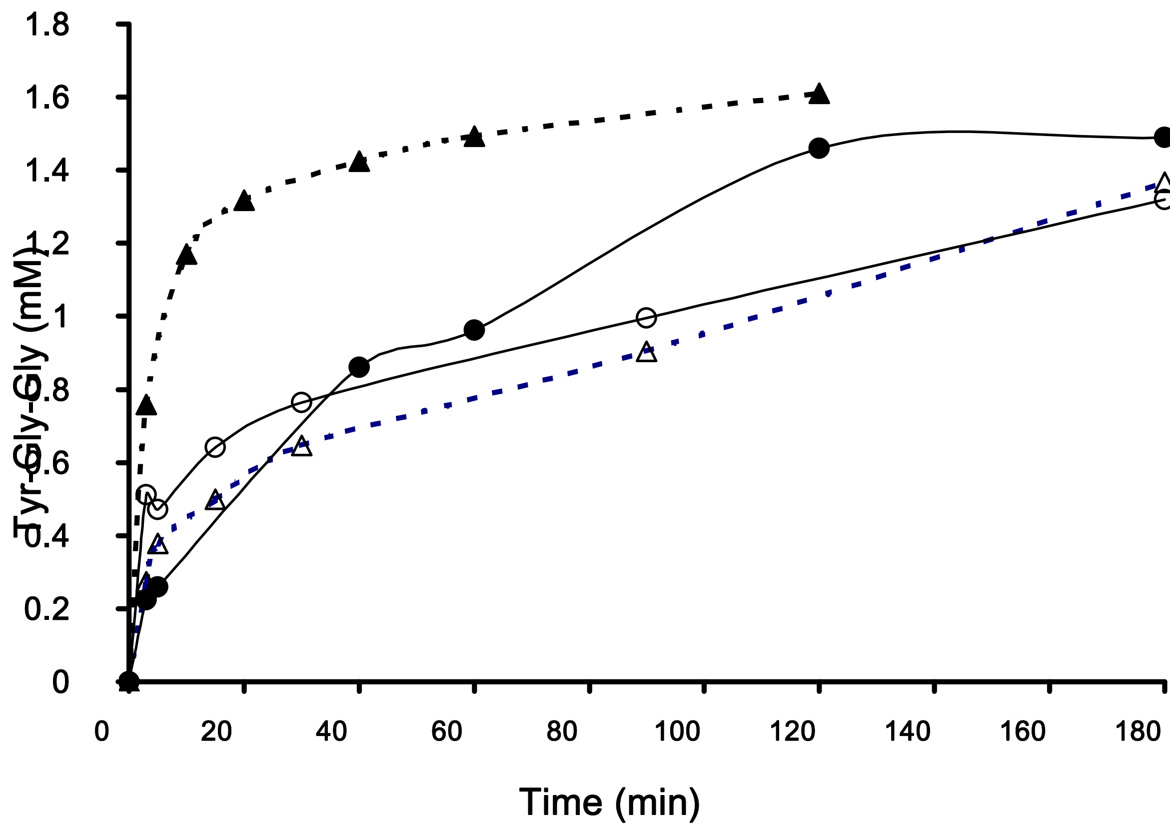

Fig. 2

C

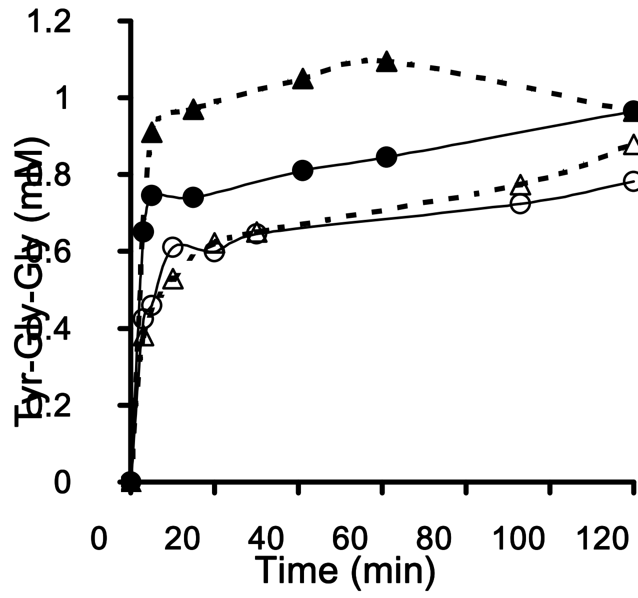

Fig. 2

D