RESEARCH LETTER

Esterase SeE of *Streptococcus equi* ssp. *equi* is a novel nonspecific carboxylic ester hydrolase

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Abstract

Extracellular carboxylic ester hydrolases are produced by many bacterial pathogens and have been shown recently to be important for virulence of some pathogens. However, these hydrolases are poorly characterized in enzymatic activity. This study prepared and characterized the secreted ester hydrolase of *Streptococcus equi* ssp. *equi* (designated SeE for *S. equi* esterase). SeE hydrolyzes ethyl acetate, acetylsalicylic acid, and tributyrin but not ethyl butyrate. This substrate specificity pattern does not match those of the three conventional types of nonspecific carboxylic ester hydrolases (carboxylesterases, arylesterases, and acetylesterases). To determine whether SeE has lipase activity, a number of triglycerides and vinyl esters were tested in SeE-catalyzed hydrolysis. SeE does not hydrolyze triglycerides and vinyl esters of long-chain carboxylic acids nor display interfacial activation, indicating that SeE is not a lipase. Like the conventional carboxylesterases, SeE is inhibited by di-isopropylfluorophosphate. These findings indicate that SeE is a novel carboxylesterase with optimal activity for acetyl esters.

Introduction

Carboxylic ester hydrolases are a diverse group of enzymes that hydrolyze carboxylic esters. They can be divided into nonspecific and specific carboxylic ester hydrolases, and the former can be divided into lipases and esterases. Lipases catalyze hydrolysis of both short-chain (water-soluble) and long-chain (water-insoluble) triglycerides and are usually interfacially activated with an abrupt increase in activity when substrates form emulsions. On the other hand, esterases act on only short-chain triglycerides in solution and are not interfacially activated in substrate emulsions. Besides triglycerides, vinyl esters have also been used to distinguish esterases from lipases. Unlike lipases, esterases are inactive against long-chain vinyl esters in solution or emulsion (Chahinian *et al.*, 2002).

Nonspecific carboxylic ester hydrolases can also be classified into carboxylesterase, arylesterase and acetylesterase based on substrate specificity using ethyl acetate, ethyl butyrate, tributyrin and phenyl acetate as well as their sensitivity to di-isopropylfluorophosphate (DFP) (Whitaker, 1972). Phenyl acetate is hydrolyzed by all the three types of nonspecific carboxylic ester hydrolases, while ethyl acetate is hydrolyzed by carboxylesterases and acetylesterases but not by arylesterases. Ethyl butyrate and tributyrin are hydrolyzed only by carboxylesterases, and carboxylesterases and some arylesterases are inhibited by DFP (Whitaker, 1972; Fenster *et al.*, 2003).

It has been known that esterases are widespread in nonpathogenic and pathogenic bacteria (Stock *et al.*, 1961; Bornscheuer, 2002). However, the roles of esterases in virulence and pathogenesis of pathogenic bacteria are largely unknown. Active and passive immunizations with the secreted esterase of Group A *Streptococcus* protect mice against subcutaneous infection of Group A Streptococci (Liu *et al.*, 2007). A cell wall-anchored carboxylesterase is required for virulence in *Mycobacterium tuberculosis* (Lun & Bishai, 2007), and a putative esterase was among the genes required for lung infection in mice caused by *Streptococcus pneumoniae* in a large-scale screen (Hava & Camilli, 2002). Nevertheless, the enzymatic activities of these extracellular esterases have not been characterized in detail, although an intracellular esterase, Rv1399c, of *M. tuberculosis* has been characterized (Canaan *et al.*, 2004). The horse pathogen *Streptococcus equi* ssp. *equi*, which causes equine strangles (Harrington *et al.*, 2002), has a homologue of the secreted

esterase of Group A *Streptococcus* (designated SeE for *S. equi* esterase). We prepared recombinant SeE and characterized its enzymatic activity. Our results indicate that SeE is a novel nonspecific carboxylic ester hydrolase.

Materials and methods

Materials and bacterial strain

Tripropionin was purchased from TCI America (Portland, OR). Vinyl propionin, vinyl butyrate, vinyl laurate, tributyrin, trioctanoin and lipase from *Mucor meihei* (5350 U mg^{-1} solid) were purchased from Sigma (St. Louis, MO). Acetylsalicylic acid, triacetin, and ethyl butyrate were purchased from Fisher Scientific (Fair Lawn, NJ). *Streptococcus equi* strain SEM1 has been described (Liu *et al.*, 2008).

Gene cloning

The *see* gene encoding the secreted esterase of *S. equi* was PCR cloned from strain SEM1 using primers 5′-ACCAT GGGCACGCGATCCTGGAAAAGCTG-3′ and 5′-CGAAT TCTTATTTTTGGGGTTCGTACTC-3′. The PCR product was digested with EcoRI and NcoI and was ligated into pET-His (Lei *et al.*, 2003) at the EcoRI and NcoI sites to yield the plasmid pSEE. Recombinant SeE made from this construct had 12 amino acid residues, MHHHHHHLETMG, fused to the second amino acid residue, ^{34}Thr, of mature SeE. The cloned gene was sequenced to rule out spurious mutations.

Purification of recombinant SeE

Recombinant SeE was expressed and purified from *Escherichia coli* strain BL21 containing pSEE. The bacteria were grown in 6 L of Luria–Bertani (LB) broth supplemented with 100 mg ampicillin L^{-1} at 37 °C. When the $OD_{600\,nm}$ of the culture was about 0.5, 0.5 mM isopropyl-β-D-thiogalactopyronoside was added to induce SeE production. After 10 h of induction, bacteria were harvested by centrifugation.

The bacterial pellet obtained was suspended in 80 mL of 20 mM Tris-HCl, pH 8.0, sonicated on ice for 20 min and centrifuged. The lysate was adjusted to 0.5 M NaCl and loaded onto a Ni-nitrilotriacetic acid agarose column (2.5 cm × 3 cm). The column was washed with 50 mL of 20 mM Tris-HCl containing 0.5 M NaCl and eluted with a 100-mL gradient of 0–75 mM imidazole. Fractions containing SeE were pooled and dialyzed against 3 L of 20 mM Tris-HCl, and the dialyzed sample was loaded onto a diethylaminoethyl sepharose column (2.5 cm × 3 cm) that was eluted with a 110-mL gradient of 0–50 mM NaCl. Fractions containing SeE with > 95% purity were pooled and dialyzed against 20 mM Tris-HCl.

SeE activity assay

The enzymatic activity of SeE was determined with potentiometric titration using a pH-stat (Model 360, Denver Instrument), as described previously with minor modifications (Chahinian *et al.*, 2002). Titration with 0.02 N NaOH was performed at 25 °C for 3 min in 25 mL of 2.0 mM Tris-HCl, pH 7.6, containing SeE and substrate at specified concentrations. Hydrolysis rate was measured from the slope of the plot of the volume of NaOH added vs. time. Enzyme activity was defined as micromoles of acid formed min^{-1} mg^{-1} SeE protein.

Inhibition of SeE activity by DFP

SeE at 0.26 mg mL^{-1} in 100 μL of 2 mM Tris-HCl, pH 7.4, was incubated with DFP at concentrations from 0.0 to 100 μM at 37 °C for 30 min, and the remaining activity of the treated SeE was determined using 100 mM vinyl propionate as described above.

SeE stability at acidic and basic pH

SeE at 0.26 mg mL^{-1} was incubated at 25 °C for 1 h in the buffers at pH from 4 to 9 that were same as those used by Canaan *et al.* (2004), and the remaining activity of the treated SeE was determined using 100 mM vinyl propionate as described above.

Temperature dependence of SeE activity

To evaluate the effects of temperature on SeE activity and stability, SeE activity was determined at 10, 20, 30, 40, 50 and 60 °C for 5 min using 100 mM vinyl propionate as described above.

Nucleotide sequence accession number

The GenBank accession number for the nucleotide sequence of the *S. equi see* gene is EU938321.

Results

SeE gene and protein

A BLAST search of available *S. equi* genome (http://www.sanger.ac.uk/Projects/S_equi) with the *sse* gene of Group A *Streptococcus* identified the esterase gene of *S. equi*, *see*, which encodes a 345-amino-acid protein with an inferred molecular mass of 39 358 Da. The inferred protein has a putative 32-amino-acid secretion signal sequence. The Sse and SeE proteins share 62% sequence identity (72% similarity). To obtain recombinant SeE, the *see* gene was cloned, and recombinant SeE protein was overexpressed in *E. coli* and purified to > 95% purity as determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (data not shown). SeE was detected in

the culture supernatant of all five *S. equi* strains tested and had specific antibody in convalescent sera of all five horses with strangles (data not shown), indicating that SeE is produced *in vitro* and *in vivo*.

SeE does not belong to the conventional three types of nonspecific carboxylic ester hydrolases

Differential specificities of esterases for substrates ethyl acetate, phenyl acetate, ethyl butyrate and tributyrin have been used to divide nonspecific carboxylic ester hydrolases into groups of carboxylesterases, arylesterases and acetylesterases. We used acetylsalicylic acid, a derivative of phenyl acetate, and the other substrates to determine whether SeE is a member of one of these esterase families. SeE-catalyzed ester hydrolysis was monitored with potentiometric titration using a pH-stat in which NaOH was automatically pumped in to neutralize released acid and keep pH of the reaction solution constant. After SeE protein was mixed with 25 mM ethyl acetate, 0.02 M NaOH was pumped in at a constant rate of 0.06 mL min^{-1}, the slope of the plot of the accumulative NaOH volume vs. time (Fig. 1a). As expected, no hydrolysis was observed in a control reaction without SeE (Fig. 1a). These results indicate that SeE can catalyze hydrolysis of ethyl acetate. From the rate of NaOH addition, an observed reaction rate was calculated to be that 23 μmol ethyl acetate was hydrolyzed per minute by 1 mg SeE. The reaction was repeated at other ethyl acetate concentrations, and the observed rate increased hyperbolically with increasing substrate concentration (Fig. 1b). Double-reciprocal plotting analysis of the data in Fig. 1b indicates that the reaction catalyzed by SeE follows the Michaelis–Menten model (Fig. 1c). According to the slope and intercept on the *y*-axis in Fig. 1c, the Michaelis constant (K_m) and specific activity of SeE were calculated to be 56 mM and 68 μmol min^{-1} mg^{-1}, respectively (Table 1). Because arylesterases do not hydrolyze ethyl acetate, the ability of SeE to hydrolyze ethyl acetate indicates that it is not an arylesterase.

We next tested the activity of SeE on the other substrates. SeE cannot hydrolyze ethyl butyrate (Fig. 2a). Because carboxylesterases, but not acetylesterases, hydrolyze ethyl butyrate, the inability of SeE to hydrolyze ethyl butyrate indicates that SeE is not a member of the conventional carboxylesterase. This result also suggests that SeE may be an acetylesterase, which cannot hydrolyze tributyrin. However, SeE hydrolyzes tributyrin with a specific activity of 3.4 μmol min^{-1} mg^{-1} using an optimal substrate concentration (Fig. 2a). These results indicate that SeE is not an acetylesterase. Thus, SeE does not belong to any of the three conventional nonspecific carboxylic ester hydrolases.

Ability to hydrolyze phenyl acetate appears to be a common feature of nonspecific carboxylic ester hydrolases because all the three types of nonspecific carboxylic ester hydrolases can hydrolyze phenyl acetate. Therefore, we tested whether SeE also hydrolyzes phenyl acetate-type ester. SeE can hydrolyze acetylsalicylic acid, a derivative of phenyl acetate. The reaction shows a hyperbolic relationship between the observed rate of hydrolysis and substrate concentration (Fig. 2b) and follows the Michaelis–Menten model with a K_m value of 3.3 mM and a specific activity of

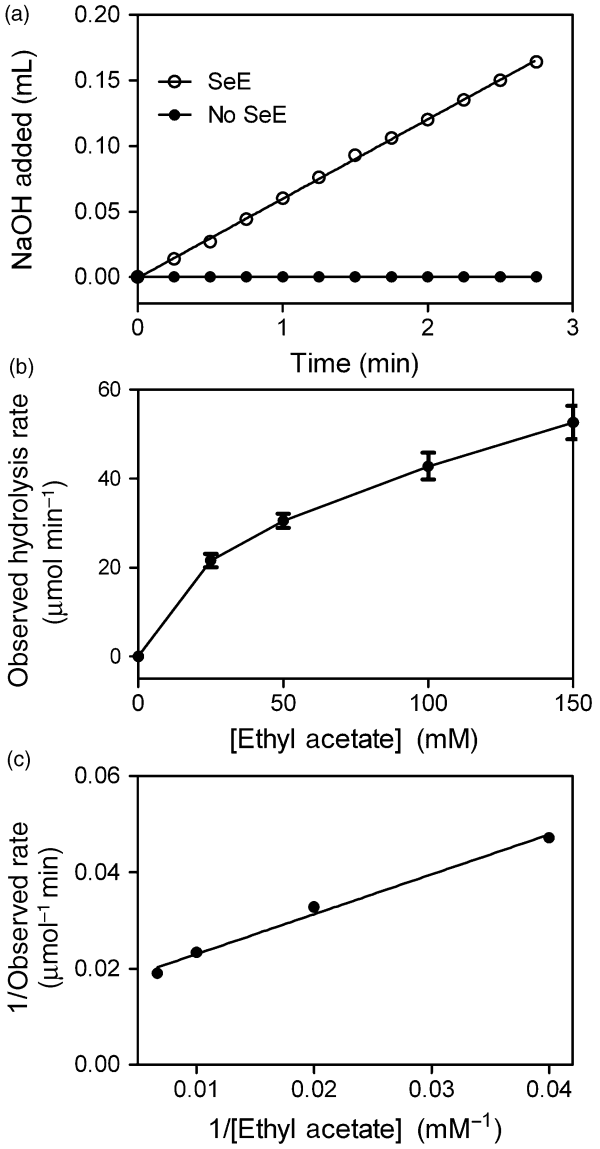

Fig. 1. SeE-catalyzed hydrolysis of ethyl acetate. (a) Titration of the released acetic acid with 0.02 N NaOH in a reaction of 25 mM ethyl acetate in the absence (•) or presence (○) of 52 μg SeE in 25 mL of 2 mM Tris-HCl buffer, pH 7.6, at 25 °C using a pH-stat. Presented is the accumulative volume of NaOH added as a function of time. (b) Plotting of hydrolysis rate vs. ethyl acetate concentration. The rates were calculated from the slope of the curve in (a) and similar experiments at the other ethyl acetate concentrations. (c) Double reciprocal plotting of the data in (b).

Table 1. Specific activities and K_m of SeE and *Mucor meihei* lipase against various substrates

	SeE		Lipase
Substrates	Specific activity (μmol min^{-1} mg^{-1})	K_m (mM)	Specific activity (μmol min^{-1} mg^{-1})
Ethyl acetate	68*	56	2†
Ethyl butyrate	0		13‡
Tributyrin	3†		133†
Acetylsalicylic acid	44*	3	1†
Triacetin	513*	203	2*
Tripropionin	149* (73†)	29	22‡
Trioctanoin	0		111‡
Vinyl propionate	815*	68	573†
Vinyl butyrate	24†		412‡
Vinyl laurate	0		13‡
Acetylcholine	0		0

*The value was obtained by using double-reciprocal analysis of enzyme activity at substrate concentrations lower than solubility.
†The activity was measured at optimal substrate concentration.
‡The activity was determined at 100 mM substrate.

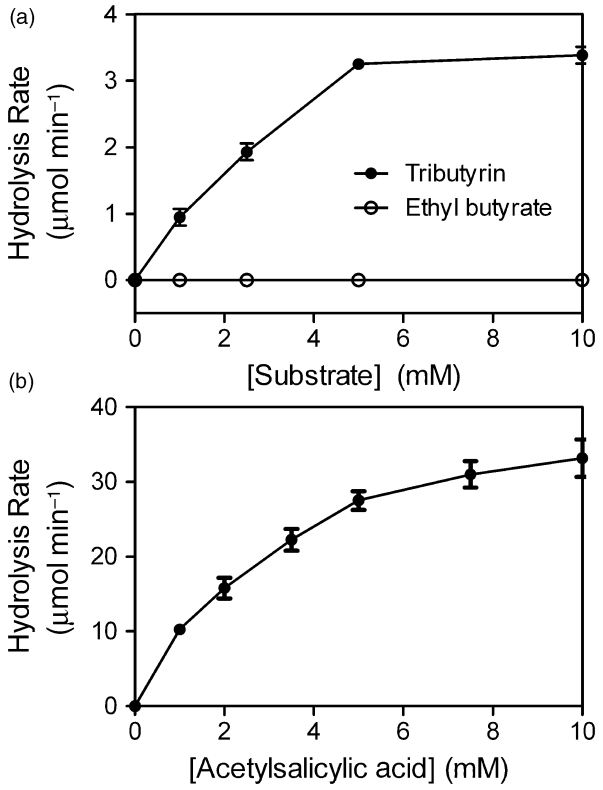

Fig. 2. SeE hydrolyzes tributyrin (a) and acetylsalicylic acid (b) but not ethyl butyrate (a). The hydrolysis assays were performed as in Fig. 1, and the hydrolysis rates of the indicated reactions are presented as a function of substrate concentration.

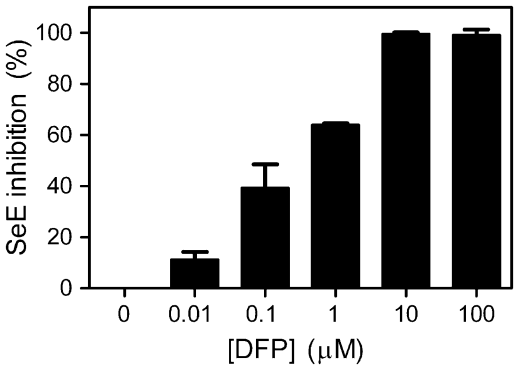

Fig. 3. Inhibition of SeE activity by DFP. SeE was incubated with DFP at 37 °C for 30 min, and the remaining activity of the treated SeE was determined using 100 mM vinyl propionate. Presented are the percentages of inhibition at the indicated DFP concentrations.

44 μmol min^{-1} mg^{-1}. This result supports the hypothesis that SeE is a member of nonspecific carboxylic ester hydrolases.

Inhibition of SeE activity by DFP

DFP is a potent inhibitor of carboxylesterases and some arylesterases but not acetylesterases (Whitaker, 1972; Fenster *et al.*, 2003). Whether SeE is inhibited by DFP was tested to further confirm that SeE is not an acetylesterase. SeE was incubated with DFP from 0 to 100 μM for 30 min, and the SeE activity was determined using vinyl propionate as a substrate. The activity of SeE was inhibited in a dose-dependent manner by DFP at $< 10\,\mu$M, and SeE was completely inhibited by DFP at $\geq 10\,\mu$M (Fig. 3). These results confirm that SeE is not an acetylesterase and suggest that SeE is more like a carboxylesterase.

SeE does not have lipase activity

Nonspecific carboxylic ester hydrolases can be either esterases or lipases. One distinction between esterases and lipases is that lipases, but not esterases, display interfacial activation when substrates turn from a solution to emulsion. To use this criterion to test whether SeE is a lipase, we compared the activities of SeE and *M. meihei* lipase on tripropionin, which has the solubility of 12.5 mM in water and forms emulsion at $>$ 12.5 mM. *Mucor meihei* lipase had low activities to hydrolyze tripropionin at up to 12.5 mM but had much higher activities when tripropionin was at $>$ 12.5 mM (Fig. 4). In contrast, SeE activity increased hyperbolically with increasing substrate concentration and reached its maximal value at tripropionin concentration slightly beyond 12.5 mM without obvious interfacial activation. Similar results were obtained using vinyl propionate (data not shown). These results suggest that SeE is not a lipase.

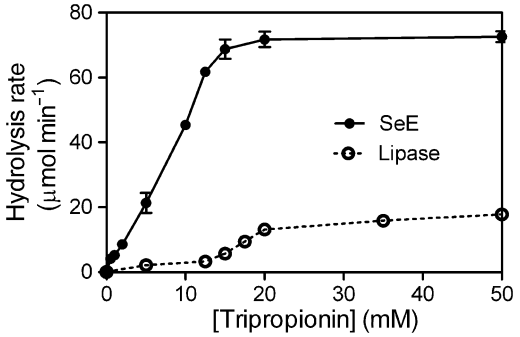

Fig. 4. Lack of interfacial activation in SeE-catalyzed hydrolysis of vinyl propionate. The hydrolysis reactions of tripropionin at various concentrations in the presence of 220 μg SeE (•) or 375 μg *Mucor meihei* lipase (○) were performed as described in Fig. 1. The hydrolysis rates are presented as a function of substrate concentration.

Another distinction between esterases and lipases is that esterases hydrolyze short-chain triglycerides and vinyl esters, whereas lipases are active for both short- and long-chain triglycerides and vinyl esters. We tested the activities of SeE and *M. meihei* lipase for the following substrates: triacetin, tripropionin, trioctanoin, vinyl propionate, vinyl butyrate and vinyl laurate. SeE is active for the short-chain substrates triacetin, tripropionin, tributyrin, vinyl propionate and vinyl butyrate but inactive for the long-chain substrates trioctanoin and vinyl laurate (Table 1). In contrast, *M. meihei* lipase is active for all these substrates. These results further confirm that SeE is not a lipase.

Specific carboxylic ester hydrolases include phopholipases and acetylcholinesterases. Because SeE has no activity for the long-chain esters, SeE is not expected to be a phospholipase. SeE was found to be inactive for acetylcholine (Table 1). This result further supports the hypothesis that SeE is a nonspecific carboxylic ester hydrolase.

Stability at acidic and basic pH

The activities of SeE after 1-h incubation at pH 4 and 5 were < 20% of the activity of SeE after 1-h incubation at pH 7, whereas only a slight decrease was observed after incubation at pH 8 and 9 (Fig. 5a), indicating that SeE is not stable in acidic solutions.

Temperature dependence of SeE activity

Time courses of NaOH titration for SeE activity at different temperatures are shown in Fig. 5b. The cumulative NaOH added as a function of time at different temperatures shows that the hydrolysis rate increased with temperature with an optimal temperature at 40 °C, indicating that higher temperature enhances reaction rates. However, the reaction rates dramatically slowed down with time at 50 and 60 °C, indicating that SeE was rapidly inactivated at these temperatures.

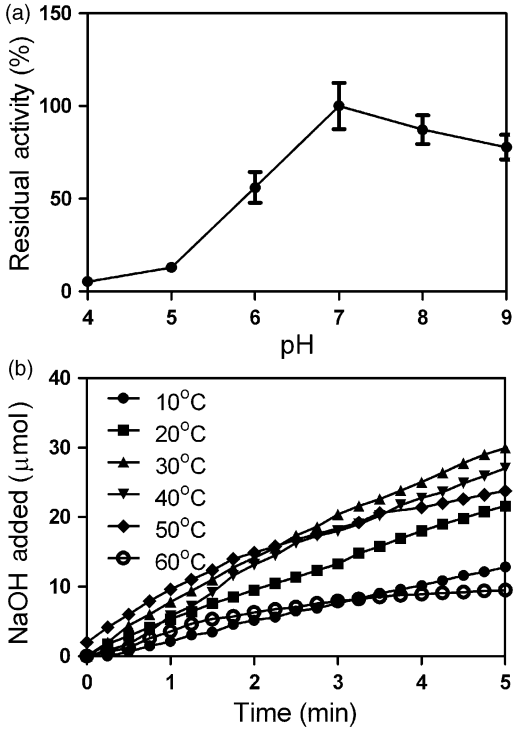

Fig. 5. (a) Instability of SeE at low pH. SeE was incubated at 25 °C for 1 h in the buffers at pH from 4 to 9, and the remaining activity of SeE was determined using 100 mM vinyl propionate. (b) Effect of temperature on SeE activity. SeE activity was determined at 10, 20, 30, 40, 50, and 60 °C for 5 min using 100 mM vinyl propionate. Shown is the accumulative NaOH added as a function of time.

Discussion

Many bacterial pathogens produce extracellular carboxylic ester hydrolases. Some recent publications report that these proteins are important for virulence (Hava & Camilli, 2002; Liu *et al.*, 2007; Lun & Bishai, 2007). These proteins are poorly characterized in enzymatic activity, and whether these proteins are different from other characterized carboxylic ester hydrolases is not known. We have characterized the enzymatic activity of SeE by *S. equi*. We found that SeE does not belong to any of the three conventional families of nonspecific carboxylic ester hydrolases nor has lipase activity. Our results thus identify SeE as a secreted novel nonspecific carboxylic esterase.

SeE is ruled out as a member of arylesterases or acetylesterases according to the observed substrate specificity. Even though SeE is inhibited by DFP-like carboxylesterases, unlike these conventional carboxylesterases, SeE does not hydrolyze ethyl butyrate. SeE hydrolyzes many carboxylic esters of aliphatic and aromatic alcohols like conventional carboxylesterases, suggesting that SeE is similar to carboxylesterases.

The inability of SeE to hydrolyze ethyl butyrate is a surprise. A possible explanation for this outcome is that the binding site for the acyl group of esters is more suitable to ethyl group in SeE. If this is true, the ethyl group of ethyl butyrate, the alcohol moiety, may occupy the site for the acyl group, blocking the catalysis. SeE is active against vinyl butyrate, and the specific activity of SeE for triacetin is 170 times higher than that for tributyrin, supporting this interpretation. In this sense, SeE is similar to acetylesterases. However, SeE is not a typical acetylesterase because it is inhibited by DFP. Our results thus suggest that SeE is a carboxylesterase with optimal activity against acetyl esters. This enzymatic feature might have been evolved for possible function of SeE in modification of the host's upper respiratory tract during *S. equi* infection. *Streptococcus equi* causes infection of the upper respiratory tract, which is covered by mucosa. The major component of the mucosa is mucin, a glycoprotein with O-acetylated sialic acids at the outermost ends of glycans (Reuter *et al.*, 1983; Tiralongo *et al.*, 2000). SeE may modify the mucosa by hydrolyzing the O-acetyl group of mucin.

Because SeE is inhibited by DFP, a serine-reacting compound (Whitaker, 1972), SeE must have a seryl hydroxyl group in its active site, a possibility supported by the fact that SeE shares high sequence identity with the serine esterase Sse of group A *Streptococcus* (Liu *et al.*, 2007). Both SeE and Sse contains the GXSXG motif of serine esterases (Okazaki *et al.*, 2006), and the serine residue of the GXSXG motif in Sse is indeed critical for its esterase activity (Liu *et al.*, 2007).

SeE is very similar to *M. tuberculosis* esterase Rv1399c (Canaan *et al.*, 2004) in the instability to acidic pH and high temperatures. However, the pocket for acyl binding in SeE is apparently smaller than that in Rv1399c because SeE has higher activities for acetyl esters than those for the same types of butyryl esters, whereas Rv1399c has higher activities for propionyl and butyryl esters than acetyl esters (Canaan *et al.*, 2004). This again supports the idea that SeE primarily targets acetyl esters.

Acknowledgements

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