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New Horizon of Organosilicon Chemistry

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Enzyme mediated silicon–oxygen bond formation; the use of *Rhizopus oryzae* lipase, lysozyme and phytase under mild conditions†

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The potential for expanding the variety of enzymic methods for siloxane bond formation is explored. Three enzymes, *Rhizopus oryzae* lipase (ROL), lysozyme and phytase are reported to catalyse the condensation of the model compound, trimethylsilanol, formed *in situ* from trimethylethoxysilane, to produce hexamethyldisiloxane in aqueous media at 25 °C and pH 7. Thermal denaturation and reactant inhibition experiments were conducted to better understand the catalytic role of these enzyme candidates. It was found that enzyme activities were significantly reduced following thermal treatment, suggesting a potential key-role of the enzyme active sites in the catalysis. Similarly, residue-specific modification of the key-amino acids believed to participate in the ROL catalysis also had a significant effect on the silicon bio-catalysis, indicating that the catalytic triad of the lipase may be involved during the enzyme-mediated formation of the silicon–oxygen bond. *E. coli* phytase was found to be particularly effective at catalysing the condensation of trimethylsilanol in a predominantly organic medium consisting of 95% acetonitrile and 5% water. Whereas the use of enzymes in silicon chemistry is still very much a developing and frontier activity, the results presented herein give some grounds for optimism that the variety of enzyme mediated reactions will continue to increase and may one day become a routine element in the portfolio of the synthetic silicon chemist.

Introduction

Silicon oxygen bonds, and particularly disiloxane bonds, Si–O–Si, are ubiquitous in the biosphere. Silica in organisms such as diatoms comprises a covalently bonded framework largely composed of Si–O–Si bonds with some Si–OH bonds. Synthetic materials such as silicones and related compounds have backbones of long chains, rings or cages of silicon–oxygen bonds.[1]

In processes collectively called biosilicification, structural silica is produced in diatoms and other similar organisms from the low concentration of silicic acid in the aqueous environment. The mechanisms of these Si–O–Si bond forming reactions are not yet completely understood but polypeptides and proteins have been shown to participate actively in such biosilicification processes[2,3], In silica biosynthesis, proteins believed to be responsible for the deposition of silica in two different families, diatoms[4] and marine sponges[3,4] have been isolated and partially characterized. In particular, Morse and coworkers[3] have isolated proteins (silicateins) from the *T. Aurantia* marine sponge that catalyze the *in vitro* polycondensation of tetraethoxysilane (TEOS). Based on site-directed mutagenesis[5] the enzymatic active site of these silicatein proteins was found to catalyze the hydrolysis and/or condensation of alkoxysilanes during biosilicification reactions.

Given the mild conditions of biological Si–O bond formation it is of major interest to attempt to transfer biological methodologies into inorganic and organometallic chemistry to devise new, greener, more sustainable and more economical ways to synthesise industrially important organosilicon compounds such as silicones. Our current interest in this emerging field is the discovery and analysis of the mode of action of novel enzymes that can facilitate siloxane bond formation under mild conditions. The eventual aim is to develop alternative means of silicone and silsesquioxane production, particularly of 'designed' materials—for example stereoregular silicone polymers—where the usual equilibration reactions prevent their synthesis. This is an emerging field that is, to date, not well developed. By contrast there is already significant interest in organosilicon compounds in biotechnological and medicinal applications and the subject has also recently been reviewed.[6] Since the 1970's Tacke has led the field in modifying the action of selected drugs by the strategic replacement of carbon by silicon, for which he has coined the term 'silicon switch'.[7,8] Thus, there is a rapidly growing interest in silicon compounds in a wide variety of biological, medicinal and biotechnological contexts.

Several examples of enzyme-mediated reactions involving organosilicon compounds have been reported; however, most of these transformations do not directly involve the silicon atom. For example lipases have been used to study the selective synthesis of organosilicon esters under mild reaction conditions.[9] In another example, optically active silylmethanol derivatives were prepared by esterification with hydrolases[10] and papain was found to be particularly enantioselective. Hydrolases have also been used successfully to catalyze the esterification of trimethylsilylpropanol isomers with 5-phenylpentanoic acid under mild conditions.[11] Since β-hydroxyalkylsilanes are unstable in the presence of acid

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† Electronic supplementary information (ESI) available: Sequence alignment for phytases *Aspergillus ficuum*, *Aspergillus niger* and *E. coli* phytases. See DOI: 10.1039/c0dt00151a

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or base, the mild conditions of the enzymic reaction allowed the ester to be formed without decomposition. Acrylate modification of organosilicon compounds was achieved using hydrolases such as esterases, lipases and proteases.[12] For example, Novozyme® 435 (N435, a commercial source of *Candida antarctica* lipase B immobilized on acrylic resin beads) was used to catalyze the esterification of a silicone polyether. Advantages of the methodology included elimination of unwanted side reactions and improved safety of the work-up procedure as opposed to conventional routes. Enzymes were used for the synthesis of carbohydrate-functional amphiphilic siloxanes. These "sweet silicones"[13] possess exceptional properties, depending on the attached carbohydrate. For example, they can be used as surfactants and emulsifiers, adhesion promoters, or chiral templates. Potato phosphorylase and α-D-glucose-1-phosphate were used in a citrate buffer at 37 °C to extend a synthetic maltoheptose-functionalized PDMS. More recently, Gross and co-workers[14] reported the synthesis of a PDMS-ethyl glucoside copolymer using an immobilized lipase (N435). Hydrolase enzymes were used to enable the synthesis of organosilicon amides under mild reaction conditions.[15] Lipoprotein lipase A and cholesterol esterase type A from *Pseudomonas sp.* were determined to have the highest activity for catalyzing the formation of an amide bond between octanoic acid and aminomethyltrimethylsilane in wet 2,2,4-trimethylpentane at 30 °C after two days of reaction.

There are cases where the silicon atom is directly implicated in an enzymic reaction. The first reported case of direct siloxane biocatalysis concerned the hydrolysis of silatranes.[16] An esterase obtained from the yeast *Rhodotorula mucilaginosa* was found to catalyze the complete hydrolysis of the silatranes in Tris–HCl buffer (pH 6.8) over 4 h at 30 °C. Deactivated protein, after thermal denaturation or treatment with EDTA, SDS and $HgCl_2$, resulted in loss of activity, suggesting a catalytic role for the enzyme. In another example, Nishino[17] reported an enzymatic silicone oligomerization catalyzed by a lipid-coated lipase. *Rhizopus delemar* lipase was found to be able to catalyze, with proposed active site participation, the polycondensation of diethoxydimethylsilane in *iso*octane containing 2 wt% water at 40 °C over 20 h.

Our group has recently reported[18] the first detailed, unambiguous example of biocatalysis at silicon, showing how the active site of trypsin, a serine protease enzyme, was able to catalyze siloxane bond formation under mild conditions during the *in vitro* hydrolysis and condensation of model, monofunctional alkoxysilanes. The reaction and conditions were carefully chosen to allow differentiation between chemical and enzymatic catalysis. The data suggested that homologous lipase and protease enzymes also promoted the formation of siloxane bonds under the same conditions. Similarly, Zelisko and co-workers reported that certain proteases could mediate the sol–gel processing of TEOS, phenyltrimethoxysilane[19] and other organosilicon substrates.[20] We have also demonstrated that certain enzymes were able to accelerate silica deposition from silicic acid with resultant formation of silica-enzyme composites while retaining enzymatic activity.[21] By contrast, Ansorge, Schumacher and co-workers have reported[22] that the hydrolysis of a model monoalkoxysilane was not accelerated by the presence of a variety of protease and lipase enzymes, including trypsin; different reaction conditions from those reported by us, such as monomer and enzyme concentration and different analytical methods might possibly explain such apparently different biocatalytic activities.

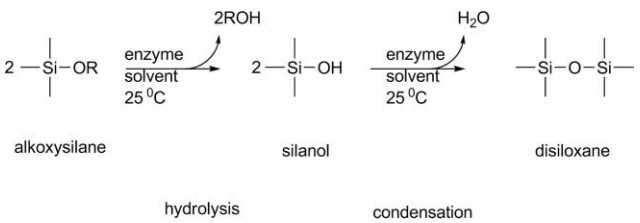
We have recently carried out a more extensive trawl of the available enzymes to screen and identify novel biocatalysts for the cleavage of Si–OR bonds and the formation of Si–O–Si bonds.[23] This led to the discovery of at least three novel potential enzymatic candidates for the biocatalysis of Si–O–Si bond formation. The enzymes that were found to be particularly good at catalyzing the reaction of interest are; a lipase from the fungus *Rhizopus oryzae* (ROL); lysozyme and several phytases from different sources.

The aim of the work presented here was to further investigate the catalytic activity of the selected enzymes in forming Si–O–Si bonds by condensation of two Si–OH units, and to elucidate the possible mechanism underlying this catalysis.

Results and discussion

Reactions in aqueous solutions

In this study of the effects of enzymes on silicon–oxygen bond formation, monofunctional silanes were chosen as the reactants to limit complexity and enable the study of the formation of molecules with only a single siloxane bond. Monoalkoxysilanes are neither obviously suitable starting materials for the preparation of silicone or silsesquioxane compounds, nor do they resemble the natural silicaceous precursor used by microorganisms (*i.e.* free silicic acid or silicates). However, this system was chosen, as previously reported,[18] as a simplified model to measure quantitatively the extent of siloxane bond formation under mild and controlled conditions. As a starting point, trimethylethoxysilane (TMES) is an ideal model compound as it has a single Si–O bond, so there are no competing reactions and the system can be followed without ambiguity. The possible products of the reaction of monoalkoxysilanes with enzymes are trimethylsilanol (TMSiOH) obtained by hydrolysis and the hexamethyldisiloxane (HMDS) obtained by condensation as shown in Scheme 1.



Scheme 1 Enzyme-catalyzed siloxane bond formation.

The hydrolysis of alkoxy- or aryloxysilanes is readily accomplished in aqueous solution at pH 7 in the presence of a wide variety of peptides and proteins and is relatively rapid and non-selective.[18] Surprisingly the condensation of silanols at pH 7 is not facile. To date only one or two enzymes have been shown to facilitate the condensation of silanols. The involvement of the enzyme active site is strongly indicated in the studies so far reported.[6,18,20] Silanol condensation taking place in the presence of enzymes at accessible rates at neutral pH, together with the even slower reverse reaction, hydrolysis of disiloxanes, is the key reaction in making silicones under non-equilibration conditions.

In this study, a variety of proteins were reacted in a buffered, pH 7, aqueous solution at 25 °C saturated with TMES, and with

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Table 1 The effect of various proteins on the hydrolysis and condensation of trimethylethoxysilane in buffered water (pH 7.0) after 24 h at 25 °C

Normalised yield (%)[a]			
Entry	TMES	TMSiOH	HMDS
negative control	10.9	88.1	1.0
bovine serum albumin	3.2	95.8	1.1
poly (L-lysine) hydrochloride	0.0	98.8	1.2
poly (allylamine) hydrochloride	3.7	93.5	2.8
Aspergillus ficuum phytase	0.0	54.6	45.4
Aspergillus niger phytase	0.0	68.2	31.8
chicken egg white lysozyme	0.0	74.9	25.1
E. coli phytase	0.0	94.4	5.6
Rhizopus oryzae lipase—ROL	0.0	38.3	61.7
Trichoderma reesei phytase	0.0	85.0	15.0

[a] Actual measured total yields of TMES, TMSiOH aand HMDS varied between 94–102%.

the excess TMES forming a separate layer; after 24 h the amounts of TMES, TMSiOH and HMDS were measured quantitatively by gas chromatography.

Although neutral pH may not be the optimum condition for the use of certain proteins, it is essential for our model studies so that acid- and base-catalyzed alkoxysilane hydrolysis and condensation is minimized and enzyme impact can be differentiated from the chemical effects of acidity or basicity.

The results are given in Table 1. Following non-specific hydrolysis of TMES, as is normally observed in the presence of a wide variety of proteins,[20] ROL, lysozyme and several phytases promoted the formation of HMDS. These proteins (enzymes) were therefore able to catalyze the condensation of the resulting TMSiOH into HMDS to a greater extent than the negative control and non-specific protein (*i.e.* BSA) or peptide (*e.g.* poly-L-lysine) reactions. Substantial hydrolysis was observed with the negative control and non-specific protein and peptide over 24 h. We were not able to differentiate between enzymatic *versus* chemically catalyzed hydrolysis over this time period. Notably, no condensation product was observed in the absence of the previously noted enzymes.

The catalytic activity of ROL and lysozyme was further investigated by conducting thermal denaturation experiments. Specifically, the enzymes were boiled for twenty minutes in Tris-buffered water (following which treatment they were inactive towards the control substrates) before adding the reactant to the cooled solution in order to determine whether the catalysis was affected by the above treatment. The results are summarized in Fig. 1.

The treatment of ROL by boiling the enzyme for twenty minutes (denaturation) drastically reduced its activity from about 62% condensation of TMSiOH to less than 5% condensation. A similar, but less dramatic, result was observed after thermal denaturation

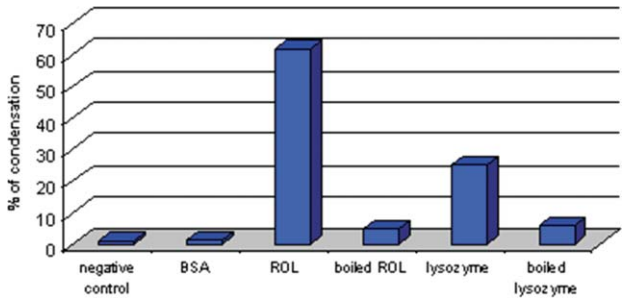


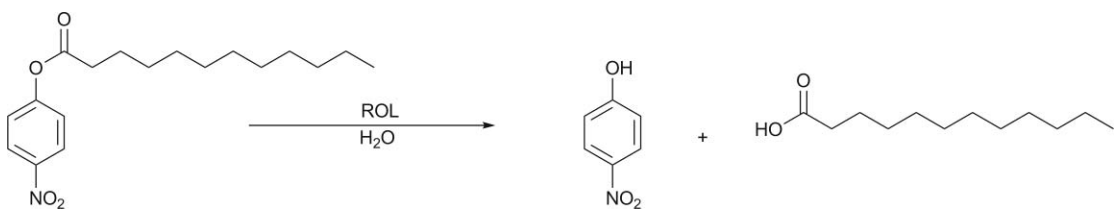
Fig. 1 ROL- and lysozyme-mediated formation of HMDS after 24 h in a neutral media at 25 °C. Substantial hydrolysis was observed with the negative control and non-specific protein and peptide over 24 h, therefore only the extent of condensation is shown.

of lysozyme where the condensation yield was reduced from 24% to around 5%. These observations strongly suggest that the tertiary structures of the two enzymes play a significant role in the catalysis of siloxane bond formation, and therefore, that the observed reactions may be active-site mediated.

Lysozyme is a small (14.3 kDa) but robust protein consisting of a single 129 amino acid polypeptide chain,[24] and containing a region of extended β-sheet with a non-polar interior. Lysozyme catalyzes the hydrolysis of the β-1,4 glycosidic bonds of the polysaccharides that comprise the peptidoglycan layer of bacterial cell walls. Hydrolysis of the glycosidic bond occurs with general acid catalysis involving the carboxylate of glutamate 35, with aspartate 52 stabilizing the transition state.[25]

Rhizopus oryzae lipase (ROL) is a small globular protein of approximately 32 kDa that exists as a monomer in solution. ROL catalyzes the hydrolysis of triglycerides to yield the free fatty acid. It favours triglycerides with alkyl chains in the region of 8 to 18 carbons long, whereas poor activity has been seen with short-chain fatty esters.[26] Synthesized as a pre-proenzyme, it contains an N-terminal signal sequence plus a prosequence that keeps it in an inactive state until it is exported from the cell.[27] The crystal structure of ROL has been solved to 2.6 Å,[28] and the protein is found be a typical α/β type protein with a central 8-stranded mixed β-pleated sheet, and with three disulfide bonds as predicted from its high homology (50% amino acid identity) with *Rhizomucor miehei* lipase.[29] The active site of lipases is similar to that in serine proteases; a triad consisting of histidine, serine and aspartic acid residues.

Reactions with ROL were further investigated, specifically to elucidate the catalytic mechanism for the lipase-catalyzed HMDS formation. First, reactant inhibition was studied by measuring the activity of ROL with the *pseudo*-natural substrate *para* nitrophenyllaurate (pNPL) shown in Scheme 2. In a spectrophotometric assay pNPL was reacted first with ROL in the absence



Scheme 2 ROL-catalyzed hydrolysis of pNPL.

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("negative control") of TMES, and subsequently in the presence of the alkoxysilane (TMES), with which it had been incubated for different time periods before addition of the pNPL. As the TMES is known to be hydrolysed rapidly, any effect on the rate of reaction can be assumed to be a result of TMSiOH interaction with the ROL active site. A lower hydrolysis rate of the pNPL in the presence of the silicon monomer would suggest a competitive binding near or in the lipase active site. Analyzing the half life ($t\frac{1}{2}$) of these reactions (based on the change in UV absorbance at 405 nm, (Fig. 2), the enzyme activity towards p-NPL decreased after various periods of incubation with the alkoxysilane. Therefore, the silicon compound seemed to interact within or near the active site region, thus causing a reduced hydrolytic activity of the lipase towards the pNPL substrate.

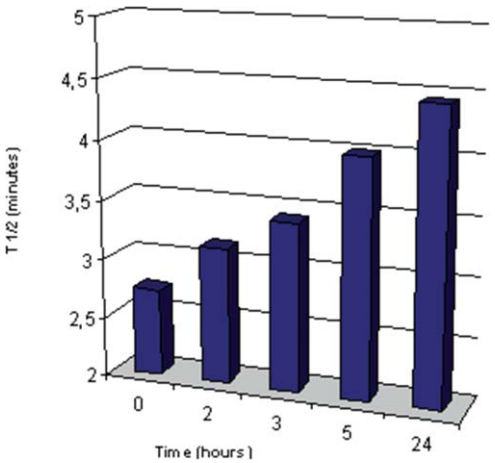

Fig. 2 Half lives of ROL-catalyzed pNPL hydrolysis after various incubation times with ethoxytrimethylsilane.

A reasonable inference is that the same active site is implicated in the silanol condensation and the pNPL hydrolysis. Recently Reetz[30] has discovered that there can be different active sites in the same enzyme with each site acting independently in different catalytic reactions. This so-called alternate-site promiscuity does not appear to be operating in this case as the TMSiOH has an effect on the ROL active site for hydrolysis. However, given the slow reduction in the rate of hydrolysis of pNPL, this may not be competitive binding either, as that would be expected to operate immediately. There are several other possible explanations for this slow inhibition. It could be slow, irreversible covalent inhibition through the formation of TMSiO-enzyme bonds at or very close to the active site. There are some potential problems with this interpretation as the condensation of TMSiOH, formed from TMES in the presence of ROL, proceeds smoothly and there is no evidence of "suicide inhibition" by TMSiOH. Further possibilities are a reversible allosteric effect[31] where the conformation of the enzyme is slowly altered by the TMSiOH binding which would have to affect the pNPL hydrolysis but not the TMSiOH condensation. It is also possible that other slow-binding inhibition mechanisms are operating.[32] We carried out some further investigation aimed at elucidating this mechanism, however it is reasonable to hypothesize that the ROL active site has a potential strong involvement in the biocatalyzed Si–O reaction.

In part, to confirm that slow enzyme inhibition is not a feature of the Si–O–Si bond forming reactions in the presence of ROL, the aqueous ROL-catalyzed hydrolysis and condensation of TMES was followed for 24 h at 25 °C (Fig. 3). The concentrations of reactant, intermediate and product were all measured over 24 h study period.

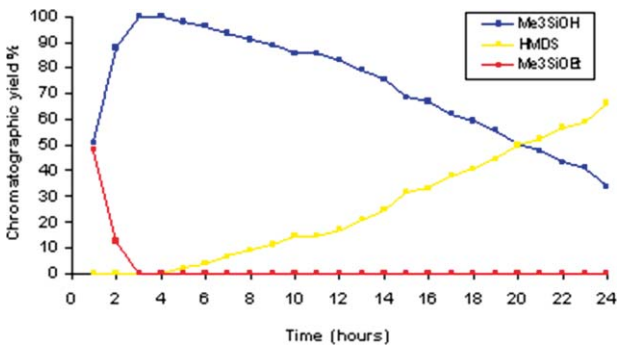

Fig. 3 Time study of the ROL-catalyzed hydrolysis and condensation of ethoxytrimethylsilane.

The hydrolysis of ethoxytrimethylsilane appeared to be complete after 4–5 h, after which hexamethyldisiloxane began to form and the yield increased smoothly. Since condensation did not occur to any great extent until the completion of hydrolysis, this may reflect the need for the silanol to achieve a sufficient concentration to act as a nucleophile on the enzyme–substrate complex. This hypothesis would be consistent with the formation of a silylated serine intermediate (enzyme–$OSiMe_3$) in or close to the lipase active site as postulated as one explanation of the results of the inhibition study (Fig. 2). The addition of TMES to the ROL showed a strong inhibition of the enzyme activity towards pNPL after 2–5 h of incubation with the alkoxysilane, corresponding to the presence of fully hydrolyzed ethoxysilane (see Fig. 3).

Further insights into the lipase action in catalysing siloxane bond formation may be obtained by selective chemical modification of certain residues in the ROL. Both serine residues and histidines may be modified chemically. In the case of ROL the serine residue in the active site acts as the nucleophilic residue, attacking the carbonyl group of its natural substrate to form a tetrahedral intermediate. The nucleophilicity of this serine is enhanced by an interaction with a histidine residue that holds it in a constrained conformation. This histidine is in turn hydrogen bonded to an aspartate residue, and it is this chain of interactions (*i.e.* catalytic triad[33]) that confers on the enzyme its catalytic properties. Covalent modification of histidines was accomplished using the reagent diethylpyrocarbonate (DEPC), and the serines were modified by treatment with diisopropylfluorophosphate (DFP).[34] The amount of reagent was calculated from the primary sequence information for each protein. Pre- and pro-protein sequences were ignored, based on the assumption that this protein is produced from its natural host organism and therefore should be correctly processed. As a result, ROL is expected to contain seven histidines and thirty serines. A large excess of modifiers (excess 10 and 100 fold molar for DFP and DEPC reactions, respectively) was added to ensure all accessible relevant residues were modified. In the modification of histidine, the nucleophilic histidine attacks the carbonate group of the DEPC to give an ethyl carboxylate modified histidine residue with the release of carbon dioxide. In the neutral Tris buffer system employed, this

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Table 2 Effect of DEPC and DFP modification of ROL on pNPL hydrolysis and HMDS formation

Reaction pNPL hydrolysis	Protein	Result Average units	% activity relative to control
	ROL control for DEPC reaction	645	
	ROL DEPC treated	26.0	4
	ROL control for DFP reaction	1636	
	ROL DFP treated	918	56

TMES hydrolysis and condensation		% yield TMSiOH	% yield HMDS	% condensation relative to ROL control
	ROL control	38	62	—
	ROL DEPC treated	64	36.	58
	ROL DFP treated	96	4	6
	Negative control	88	1	
	negative control + DEPC	99	1	

covalent adduct is stable over many hours.[35] The modification of the histidine means that it is unable to act as a base or as a nucleophile, and its participation in hydrogen bonding networks should be reduced, thus weakening the charge-relay system of the catalytic triad. Diisopropylfluorophosphate (DFP) is a very potent inhibitor of serine esterases. It covalently inactivates all the serine residues, thereby blocking the catalytic nucleophilicity of their hydroxyl functionalities. The results of the modifications of ROL on its activity towards pNPL and TMSiOH are summarised in Table 2

Although formation of HMDS was observed with the DEPC-treated ROL, the enzyme activity was halved following the treatment, suggesting at least a partial involvement of the histidines in the catalysis. DFP-treatment almost completely abolished the catalytic activity of the lipase. This result strongly highlights the essential role of serine residues in the reaction mechanism for disiloxane formation.

In summary, in aqueous solutions ROL was found to catalyze *in vitro* siloxane bond formation under mild conditions of pH and temperature. While there remains some degree of uncertainty the experiments reported here suggest that the active site of ROL, the catalytic triad, is intimately involved in the catalysis of siloxane bond formation. It also seems to be more than coincidence that the only proteins that we and others have found that catalyse silanol condensation are enzymes with some kind of hydrolase function.[6,18] However, more work is in progress to gain further insights into these examples of enzyme promiscuity involving silicon.

Reactions in organic media

In industrial contexts water is a desirable solvent but it is not always suitable. The use of organic solvents particularly in silicone fabrication is very convenient in process terms. Accordingly, the hydrolysis and condensation study of TMES was also carried out in organic media, particularly as many enzymes naturally function in hydrophobic environments, such as those in the presence of, or bound to, a membrane.[36] The TMES studies were carried out by using monophasic aqueous-organic solutions,[37] consisting of 5% v/v Tris-buffered water (pH = 7) in water-miscible organic co-solvent such as *tert*-butanol or acetonitrile. Many proteins are deactivated under these conditions and only a few retain their activity. Nevertheless, many examples of biotransformations in organic media have been reported.[37–40]

Table 3 Enzyme-catalyzed hydrolysis and condensation of trimethylethoxysilane in 5% (v/v) buffered water in *tert*-butanol after 24 h at 25 °C

Normalized yields (%)[a]			
Entry	TMES	TMSiOH	HMDS
negative control	34.7	64.4	1.0
bovine serum albumin	68.3	30.9	0.8
poly (L-lysine) hydrochloride	77.9	21.4	0.7
Chicken egg white lysozyme	10.1	78.1	11.8
Aspergillus ficuum phytase	9.7	83.4	6.9
chicken egg white lysozyme	10.1	78.1	11.8
E. coli phytase	3.8	71.0	25.2

[a] Actual measured total yields of TMES, TMSiOH aand HMDS varied between 94–102%.

Enzymes were chosen for this study that are known to be active in more hydrophobic environments and these are shown in Table 3. Following the familiar non-specific hydrolysis of the model alkoxysilane, TMES, certain enzymes (Table 3) catalyzed the condensation of the resulting TMSiOH to give HMDS to a greater extent than the negative control and non-specific proteins such as BSA or peptides. Substantial hydrolysis was observed with the negative control and a non-specific protein and peptide over 24 h. Again, we are not able to differentiate between enzymatic and chemically catalyzed hydrolysis over this time period.

Notably, no significant condensation product was observed in the absence of enzymes. As illustrated (Tables 2 and 3), several phytases promoted HMDS formation. In this case, different activities were observed, depending on the enzyme source and the solvent used.

Specifically, *A. niger*, and *A. ficuum* phytases preferentially catalyzed the reaction in water, whereas *E. coli* phytase gave a high yield of the HMDS product only in wet organic media and particularly so in wet acetonitrile. The results are summarized in Fig. 4.

The phytases are examples of histidine acid phosphatases. These show broad substrate specificity and hydrolyze phytate under

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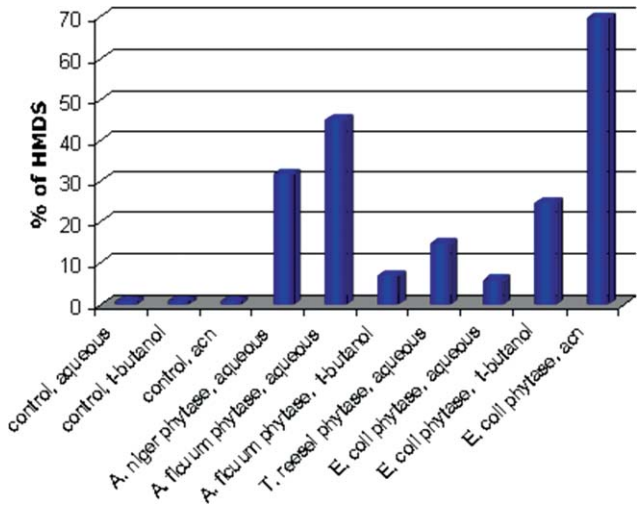

Fig. 4 Phytase-catalyzed HMDS formation after 24 h at 25 °C.

acidic pH conditions to produce myo-inositol monophosphate as the final product.[41]

The phytase from *E. coli* is a 44.6 kDa protein that consists of a highly conserved α/β-domain and a more varied α-domain with the active site located between the two domains. Mutagenesis studies alongside crystallographic analyses have been used to identify key residues within the active site that are involved in both the catalytic process and the tight binding of the substrate.[42] The active site histidine is known to be the residue responsible for catalysis through its nucleophilic attack on the phosphorus of the natural substrate. Morse has proposed a mechanism for catalysis of siloxanes bond formation by silicatein in diatoms in which nucleophilic attack on silicon by a histidine imidazole nitrogen is a key step.[3–5]

The difference in behaviour of the various phytase enzymes initially proved difficult to understand. For example in aqueous solution the *E. coli* phytase was significantly less reactive than the other phytases (Table 1). Phytase enzymes have optimum activity at acidic pH and this may account for the poor activity in neutral water in the *E. coli* phytase reactions. although that does not easily explain the difference between the enzymes. *A. niger* and *T. reesei* phytases were supplied as liquid samples, and consequently, for miscibility reasons, were only tested in aqueous solutions without investigating their activities in organic solvents. Their matrix solutions were found to be slightly acidic (pH = ~5). The acidity of the medium might have influenced the catalysis relative to the neutral buffered media (pH = 7.0) used in the reaction using *E. coli* phytase (supplied as a solid sample) if there was also residual acidity in that case. However, control reactions at the same pH of the liquid phytase matrices showed no particular condensation over 24 h (results not shown). *A. ficum* phytase (supplied as a solid sample) showed better activity in water as opposed to organic media. The differences between the aqueous activities of *E. coli* and *A. ficum* phytases are not easily explained even though not all of the phosphatase enzymes would be expected to show identical activity profiles.

We have therefore tried to understand the reasons for the different activity towards the model alkoxysilane reaction in the phytases by examining the homology of the amino acid sequences, where available, for each phytase (see ESI† for data). Data for *T. reesei* was not available.

The data for *Aspergillus ficuum*, *Aspergillus nigera* and *E.coli phytases* is given in the ESI. There is 99% homology between *Aspergillus ficuum*, and *Aspergillus nigera* which is consistent with their similar activity in aqueous solution. Surprisingly there is only a 4% homology between *Aspergillus ficuum*, *Aspergillus nigera* phytases on the one hand and *E.coli* phytase on the other. This again is fully consistent with the different reactivity patterns of *E.coli* phytase relative to the *Aspergillus* phytases. In future work to disclose the nature of the observed biocatalysis at silicon centre by phytases we aim to carry out further analyses of the probable active sites in the different phytases, possibly followed by genetic modification of the proteins.

In summary, phytases, a class of acid phosphatases, were also observed to catalyze HMDS formation under mild reaction conditions. Depending on the phytase source and solvent system, different activities were found. Notably, *E. coli* phytase gave a high yield of disiloxane product in wet organic media. This reaction may be of interest in silicone production, where the syntheses of silicon-based materials are accomplished in water-poor systems. Furthermore, silicon monomers are often insoluble in water. Therefore, the discoveries of novel biocatalysts that catalyze the siloxane bond formation in organic solvents present advantages.

Conclusion

ROL, lysozyme and four different phytase enzymes were observed to promote *in vitro* siloxane bond formation under mild reaction conditions. Thermal denaturation of ROL and lysozyme strongly suggested that the enzymatic tertiary structure may play a key-role in the catalysis. Moreover, reactant inhibition studies coupled with selective amino acid residue modification in ROL demonstrated the potential role of the active site at catalyzing the HMDS formation. The four phytases showed different activity depending on enzyme source and solvent probably due to different molecular structures and this is the subject of further research. Theses enzymes may be very useful when designing novel organosilicon biotransformations.

Whereas the use of enzymes in silicon chemistry is still very much a developing and frontier activity, the results presented herein give some grounds for optimism that the variety of enzyme mediated reactions will continue to increase and may one day become a routine element in the portfolio of the synthetic silicon chemist.

Experimental section

Materials

Aspergillus niger phytase was purchased from Interspex inc. (San Mateo, CA). *Trichoderma reesei* phytase (Finase L) was purchased from AB Enzymes (Darmstadt, Germany). *E. coli* phytase was supplied by Genencor International, Inc. (Palo Alto, CA). Acetonitrile, *Aspergillus ficum* phytase, bovine serum albumin (BSA), chicken egg white lysozyme, dodecane, hexamethyldisiloxane (HMDS), poly-(allylamine hydrochloride), poly-L-lysine, *Rhizopus oryzae* lipase, sodium chloride, *tert*-butanol, trimethylethoxysilane (TMES) and trizma pre-set crystals (pH 7.0) were purchased from Sigma Aldrich (Poole, UK). Trimethylsilanol

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(TMSiOH) was obtained from the Dow Corning Corporation (Midland, MI, USA). All the materials were used as received without further modification. Ultra high purity water (UHP) was obtained from a Milli-Q system at The Open University (Milton Keynes, UK).

Biocatalysis study

The reactions in the presence of enzymes were formulated with a 5 : 1 alkoxysilane (100 mg) to enzyme (20 mg) weight ratio in 0.5 g of the appropriate solvent system. The monophasic aqueous-organic solution consisted of 5% (v/v) Tris buffered water (pH = 7.0) in *tert*-butanol. Milli-Q water ('water') was buffered with 100 mM Tris–HCl buffer, pH 7.0 ('buffered pH 7'). Based on the estimated solubility of trimethylsilanol in water (42.56 mg mL^{-1}),[25] the concentration of trimethylethoxysilane (~200 mg mL^{-1}) saturated the aqueous media and created a two-phase reaction mixture. The time study of the *Rhizopus oryzae* lipase-catalyzed hydrolysis and condensation of trimethylethoxysilane was conducted at 25° C for defined periods of time over 24 h.

The reactions were conducted in silylated glass vials,[18a] which provided an inert surface. Trimethylsilylated vials have been shown not to contaminate the reactions with TMSiOH and HMDS.[43] Prior to analysis, the aqueous reactions were extracted with an organic solvent, while the organic reactions were directly analyzed. THF was selected as the extracting solvent, based on a previous extraction efficiency study on silicon compounds,[43] as well as on its documented use during the extraction a variety of silicones from biological matrices.[44] Sodium chloride was used to change the ionic strength of the aqueous-phase in order to separate the miscible THF-phase. The gas chromatography analysis was performed as described below. The samples were prepared at ~1% (w/w) product in a THF solution containing 1% (w/w) dodecane. The reactions were conducted in a neutral aqueous medium (pH 7.0) at 25 °C for 24 h.

The closed (screw-capped) two-phase reactions were conducted in silanizeds glass vials at 25 °C with magnetic stirring for 24 h. The reaction products were isolated and quantitatively analyzed by GC-FID. Prior to analysis, the aqueous reactions were extracted with 1 ml of THF in the presence of NaCl and filtered through a Whatman Autovial® 5 or 12 0.45-μm Teflon® filter, while the organic reactions were directly filtered and analyzed. Control reactions are defined as non-enzymatic reactions. Specifically, experiments conducted in the absence of a protein are defined as negative control reactions. Proteinaceous and peptide molecules such as bovine serum albumin and poly-L-lysine, respectively, were used to study non-specific protein/peptide catalysis.

Thermal denaturation

In the thermal denaturation experiments, the enzymes (10 mg mL^{-1}) were boiled for 20 min in 100 mM Tris–HCl buffered Milli-Q water (pH 7.0) and cooled to 25 °C before the addition of the alkoxysilane. Thereafter, the reaction mixtures were treated and analyzed in an identical manner as described above.

Diethylpyrocarbonate (DEPC) modification of histidines

Protein was added to a solution of Tris buffer (960 μL, 50 mM, pH 7.0) plus ethanol (20 μL) to give a solution of 10 mg ml^{-1}. DEPC was added (100-fold excess) and the suspension mixed by rotation (30 min, 20 °C, 120 rpm). Excess DEPC was removed by centrifugation (13,000 rpm, 2 min) and the top aqueous layer was filtered through a gel-filtration column (NAP-10, sephadex G-50) to remove any remaining unreacted DEPC. The concentration of protein in the samples was measured by UV absorbance. The activity of the protein towards pNPL hydrolysis and TMSiOH condensation was assessed *via* the relevant enzymatic activity assay and the standard HMDS formation reaction, respectively.

Diisopropylfluorophosphate (DFP) modification of serines

Protein was added to a solution of Tris buffer (980 μL, 50 mM, pH 7.0) to give a solution of 10 mg mL^{-1}. DFP was added (10-fold molar excess) and the suspension mixed by rotation (30 min, 20 °C, 120 rpm). Excess DFP and any precipitated protein were removed by centrifugation (13,000 rpm, 2 min) and the top aqueous layer was passed through a gel-filtration column (NAP-10, sephadex G-50) to remove any remaining unreacted DFP. The concentration of protein in the samples was measured by UV absorbance. The activity of the protein was assessed *via* the activity assay and HMDS formation.

Gas chromatography-flame ionization detection

The gas chromatography (GC) analysis was performed with an Agilent 6890 Series injector on an Agilent 6890 plus gas chromatograph (GC) with a flame-ionization detector (FID). The system was configured as follows: carrier gas, 99.9995% ultra high purity (UHP) helium; GC inlet, split, 250 °C, split ratio = 100 : 1, constant flow (rate = 1.0 mL min^{-1}.), Flame ionization detector at 275 °C, H_2 = 40 mL min^{-1}, Make up N_2 = 45 mL min^{-1}.; Column, HP-5MS crosslinked 5% phenylmethylsiloxane film (30 m × 0.25 mm, 0.25 μm film); GC temperature program, 50(2) → 250 (8) @ 10 °C min^{-1}, 30 min total run time; internal standard, ~1%(w/w) dodecane in THF; Data system, Agilent Technologies ChemStation. Dodecane was used as an internal standard to quantitate gravimetrically the chromatographic analyses. The samples were prepared at ~1% (w/w) product in a THF solution containing 1% (w/w) dodecane as internal standard (IS). Based on triplicate measurements, the response factors[45] for the analytes were calculated (eqn (1)), and found to be linear as a function of concentration over four orders of magnitude (*i.e.* 0.01–10% w/w).

$$RF_{analyte} = ([analyte]/Area_{analyte}) \times (Area_{IS}/[IS]) \times RF_{IS} \quad (1)$$

where, $RF_{analyte}$ = response factor for the analyte, [analyte] = concentration of the analyte, $Area_{analyte}$ = peak area of the analyte, $Area_{IS}$ = peak area of the internal standard, [IS] = concentration of the internal standard, RF_{IS} = response factor for the internal standard = 1. Subsequently, eqn (1) was used to calculate quantitatively the concentration of an analyte in the presence of an internal standard.

Spectrophotometric analysis: enzyme activity assay

4-Nitrophenyl laurate (pNPL) was used to study the lipase/esterase activity of *Rhizopus oryzae* lipase. As detailed in Table 4, the assays were formulated by transferring the reagents, with the exception of the pNPL solution (in order to avoid non-enzymatic hydrolysis), into a glass cuvette. After mixing the

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Table 4 *Rhizopus oryzae* lipase spectrophotometric enzymatic activity assay

Reagent		Volume
A	0.4 : 10 (v/v) isopropanol : 50 mM Tris–HCl buffer (pH 8.0), 0.5M $CaCl_2$	2.955 mL
B	ROL solution (0.2 mg mL^{-1}) in reagent A	15 µL
C	10 mM pNPL in acetonitrile in reagent A	30 µL

contents, background spectra were acquired at 405 nm on an Uvikon L ultraviolet–visible spectrophotometer. Subsequently, the pNPL solutions were added to the cuvettes, mixed, and the release of *para*-nitrophenol followed at 405 nm over 10 min, at 25 °C, detecting 60 points/min.

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