$P(CH_2OH)_3$– A New Coupling Reagent for the Covalent Immobilisation of Enzymes

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Tris(hydroxymethyl)phosphine, $P(CH_2OH)_3$, can be used as an effective new type of coupling agent for the immobilisation of enzymes to amino-group-containing supports.

The immobilisation of enzymes to insoluble supports has attracted considerable interest over the past decade.[1–3] Such supported catalysts facilitate simple recovery from the reaction mixture, and may offer desirable improvements in the thermal and pH stabilities and longevity of the enzyme. The most common methods for immobilisation employ amine-functionalised supports, to which the enzyme is coupled using difunctional reagents such as glutaraldehyde, carbodiimides or benzoquinone.[4] A wide range of supports have also been employed,[5] and, of these, chitosan **1** is particularly attractive[6,7] since it is inexpensive, biocompatible and its hydrophilic nature minimises the non-specific adsorption of the enzyme to the support.

Hydroxymethylphosphines containing $>P–CH_2–OH$ groups, are well known to undergo Mannich-type condensation reactions[8] at room temperature with N–H group-containing compounds, giving aminomethylphosphines, $>P–CH_2–N<$, and we recently showed that hydroxymethylphosphines can be immobilised on chitosan and chitin supports.[9] The significant stability of the $P–CH_2–N$ linkage[8] suggested that the inexpensive, water-soluble and reasonably air-stable phosphine $P(CH_2OH)_3$[8,10] might provide a novel reagent for the covalent immobilisation of enzymes, with improved binding properties over existing coupling methods. Potential advantages of using $P(CH_2OH)_3$ as a coupling agent include an increase in the number of immobilising groups, together with an improved hydrolytic stability of the resulting $P–CH_2–N$–enzyme linkages over, for example, the hydrolysable C=N–enzyme analogues formed from glutaraldehyde. Both coupling reagents ideally utilise non-essential NH_2 groups on the enzyme to minimize enzyme inactivation.

Formaldehyde-free $P(CH_2OH)_3$ was prepared *via* the literature method[10] from commercially available $P(CH_2OH)_4^+$ Cl^-, and was used as an aqueous solution. In a typical enzyme coupling experiment, chitosan flakes (Sigma) were stirred in aqueous solutions of $P(CH_2OH)_3$ for 1 h at 20 °C and subsequently rinsed three times with methanol and once with phosphate buffer (100 mmol dm^{-3}, pH 7), to give $P(CH_2OH)_3$-modified chitosan **2**. Reaction with urease (2 mg enzyme and 200 mg of **2** in 2 ml phosphate buffer at pH 7) proceeded while rotating on an orbital shaker for 6 h at 20 °C. The immobilised urease **3** was rinsed six times with buffer and dialysed until no urease could be detected in the washings.

Activity determinations of the immobilised urease were carried out in 100 mmol dm^{-3} urea in phosphate buffer at pH 7 rotating on an orbital shaker for 15 min at 20 °C. Aliquots were removed from the reaction mixture for estimation of ammonia, produced by the enzymatic hydrolysis of urea, by the phenol–hypochlorite method.[11] Control experiments were carried out in the absence of $P(CH_2OH)_3$ to determine the level of activity obtained by enzyme adsorption alone.

Moderate concentrations of $P(CH_2OH)_3$ (2.5 mg ml^{-1}) used as a coupling agent yielded enzyme activity 40% higher than that for urease adsorbed onto chitosan flakes in the absence of $P(CH_2OH)_3$ (Fig. 1). The disparity between the $P(CH_2OH)_3$-coupled and the adsorbed enzyme became greater after storing the samples for one week at 4 °C. The $P(CH_2OH)_3$-coupled urease was 60% more active than the adsorbed enzyme. Thus, the covalent binding of urease to chitosan using $P(CH_2OH)_3$ as a coupling agent seems to enhance the stability of the enzyme.

Furthermore, when the adsorbed urease chitosan flakes were washed with 1 mol dm^{-3} KCl to remove loosely bound enzyme, the activity was reduced by 50% to only 20% of the activity of the $P(CH_2OH)_3$-coupled enzyme. In contrast, the $P(CH_2OH)_3$-coupled enzyme activity was not affected by washing with 1 mol dm^{-3} KCl. As expected, the $P(CH_2OH)_3$ coupling seems to yield a strong covalent link between the enzyme and the support.

When high concentratons of $P(CH_2OH)_3$ (250 mg ml^{-1}) were used for coupling urease to chitosan, no enzyme activity was observed. This lack of any enzyme activity may be due to large numbers of PCH_2OH functional groups present on the chitosan which may lead to excessive crosslinking and subsequent inactivation of the enzyme.

This work demonstrates that $P(CH_2OH)_3$ represents a new method for enzyme immobilisation, with high retention of activity of the immobilised enzyme. The wide variety of available supports containing amine groups offers much potential for the further development of this method to

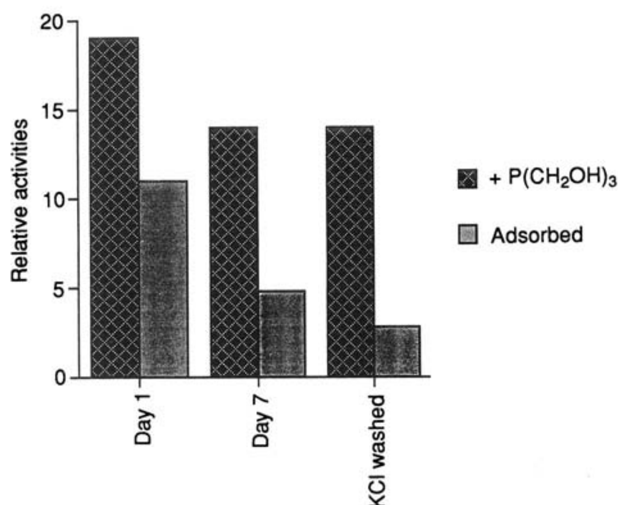
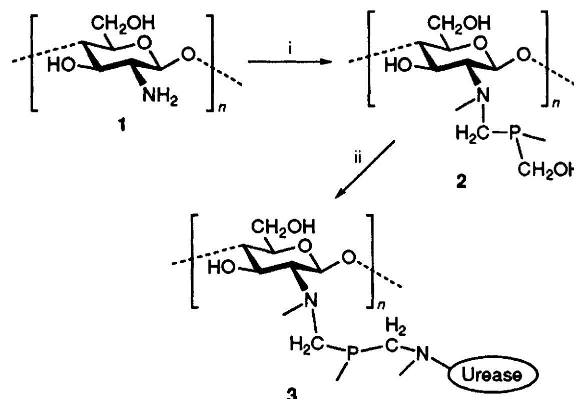


Fig. 1 Relative activities of the urease bound to the chitosan flakes by coupling with $P(CH_2OH)_3$ or adsorption. Activities were compared immediately after preparation, after one week of storage at 4 °C and after washing with 1 mol dm^{-3} aqueous KCl.



Scheme 1 *Reagents and conditions*: i, aq. $P(CH_2OH)_3$, wash; ii, urease in phosphate buffer, wash

incorporate desirable features such as flexible long-chain linkages[7,12] and biocompatibility. Studies are in progress to delineate the general applicability of this methodology.

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