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# Dipeptide synthesis in near-anhydrous organic media: Long-term stability and reusability of immobilized Alcalase

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ABSTRACT

The long-term stability and re-use of Alcalase covalently immobilized onto macroporous acrylic beads (Cov) in tetrahydrofuran (THF) were investigated. Cov can be used to synthesize dipeptides under near-anhydrous conditions in THF. Cov was incubated with and without molecular sieves (beads or powder) in THF, in order to investigate whether its stability is affected by the presence of molecular sieves. After different incubation periods, the enzyme activity was determined in an aqueous environment. In addition, Cov was repeatedly recycled to examine its reusability. Without molecular sieve beads, Cov hardly inactivated in THF. With molecular sieve beads, Cov lost activity over time. Incubated Cov samples were rotated on a blood rotator, entailing mechanical forces between Cov and the molecular sieve beads. Mechanical damage of Cov by the molecular sieve beads was found to be the main reason for the instability of Cov. During reuse, intermediate rehydration of Cov also caused a small but significant activity loss.



## 1. Introduction

Alcalase (also referred to as Subtilisin A and Subtilisin Carslberg) is a protease that may be used in the chemo-enzymatic synthesis of peptides. Peptides play an important role in the fields of health care, nutrition and cosmetics [1–5]. Alcalase can for instance catalyze the coupling of an α-amino amide and a chemically synthesized activated N-protected amino acid, *e.g.* an N-protected amino acid carbamoylmethyl (Cam) ester [6–12].

In previous work we studied the effect of water activity ($a_w$) on the rate of dipeptide synthesis [11]. It was found that a carefully chosen amount of molecular sieves was required to prevent hydrolysis, but still allow enzymatic activity; a too large amount of molecular sieves was found to dehydrate and inactivate the enzyme [11]. Furthermore, we compared different Alcalase formulations with respect to their dipeptide synthesis capability in neat organic solvent in the presence of molecular sieves (*i.e.* under near-anhydrous conditions at very low water activity). Hydration prior to drying (with anhydrous *tert*-butanol and anhydrous tetrahydrofuran (THF)) of the Alcalase formulations resulted in a significant increase in rate of the subsequent dipeptide synthesis. The most promising enzyme formulation for dipeptide synthesis in organic media on a large scale was found to be hydrated (water saturated) Alcalase covalently immobilized onto macroporous acrylic beads (in this paper abbreviated as Cov) [12]. This formulation has a reasonable activity with respect to dipeptide synthesis in near-anhydrous organic media, and, from a practical point of view, a reasonable size (150–300 μm in diameter [13]) and a uniform spherical shape. In addition, Cov features good short-term operational stability in THF as it could be reused at least twice without significant activity loss with respect to dipeptide synthesis [12], under the proviso that the enzyme was rehydrated in-between subsequent dipeptide synthesis reactions under near-anhydrous conditions.

The present study focuses on the long-term stability and extended reuse of hydrated Cov in THF. In this manuscript no dipeptide synthesis data are presented but the conditions used to study the long-term stability and extended reuse of hydrated Cov in THF are identical to the reaction conditions used in previous work to synthesize dipeptides [11,12]. We, therefore, expect that the results from this manuscript can be directly translated to dipeptide synthesis when carried out for longer periods of time.

The stability of cross-linked subtilisin crystals and native subtilisin Carlsberg in polar solvents has been studied before [14–16]. This type of data is, however, not available for the covalently immobilized Alcalase formulation (*i.e.* Cov) we are interested in. Ultimately a process design for peptide synthesis in organic media requires knowledge of and data on the operational stability of the

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enzyme formulation that was found capable of synthesizing these peptides. Extending the operational stability of an enzyme formulation by minimizing the rate of inactivation requires a mechanistic view on the causes of instability of the enzyme formulation.

The aim of our study was to investigate whether hydrated Cov is stable in dry THF and whether its stability is affected by the presence of molecular sieves. For this, Cov was incubated with and without molecular sieves (beads or powder) in THF and its aqueous activity was subsequently determined. In addition, Cov was repeatedly recycled in order to examine its reusability.

## 2. Materials and methods

### 2.1. Enzymes

Alcalase® covalently immobilized onto macroporous acrylic beads (Cov; ChiralVision, Leiden, The Netherlands) and lyophilized native protease from *Bacillus licheniformis* (also referred to as Alcalase®, Subtilisin A, and Subtilisin Carslberg; Sigma–Aldrich, Zwijndrecht, The Netherlands) were used. Cov contains the enzyme Alcalase from Novozymes Corporation [17], which is covalently immobilized onto Immobeads 150 (cross-linked copolymer of methacrylate carrying oxirane groups).

### 2.2. Chemicals

All chemicals used were reagent or analytical grade. *t*-BuOH and THF were dried over 3 Å molecular sieves, 8–12 mesh beads (Sigma–Aldrich), for ≥1 day prior to use. The molecular sieves were dried at 200 °C and *t*-BuOH was pre-heated to a liquid (40 °C) prior to use.

### 2.3. Long-term stability of Cov

Before use, Cov (40 mg) was washed with successively 1 ml of Milli-Q, to initially completely hydrate the enzyme, and 1 ml of each anhydrous *t*-BuOH and THF, to remove the excess water. A washing step involved adding washing liquid (Milli-Q, *t*-BuOH, or THF) to Cov, shaking the sample for 10 s, centrifuging the sample for 2 min at 10,000 rpm in order to facilitate the separation of the washing liquid and the enzyme formulation, and removing the washing liquid manually using a pipette. This method was analogous to the procedure used to produce propanol-rinsed enzyme preparations [18,19].

Cov (40 mg) was incubated in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm:

(1) without molecular sieves;
(2) with 10 mg of dry 3 Å molecular sieve beads per mg Cov (*in duplo*);
(3) with 10 mg of dry 3 Å molecular sieve powder (Sigma–Aldrich) per mg Cov (*in duplo*);
(4) with 10 mg pre-hydrated molecular sieve beads per mg Cov. The molecular sieve beads were pre-hydrated by adding them to a vial with Milli-Q for 10 min and subsequently removing the excess water using tissue paper;
(5) with 17.5 mg pre-hydrated molecular sieve beads per mg Cov.

Cov was also incubated with 10 mg of dry molecular sieve beads per mg Cov without spinning on a blood rotator (*in duplo*).

After different incubation periods (0–32 days), the aqueous activity of the full content of the Eppendorf tubes (Cov, THF, and molecular sieves) was analyzed (Section 2.5). For each data point in time a separate Eppendorf tube was incubated, so all time points in the figures represent independent experiments.

### 2.4. Long-term stability of native Alcalase

Native Alcalase (2 mg) was incubated in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. After different incubation periods (0–42 days), the aqueous activity of the complete content of the Eppendorf tubes (native Alcalase and THF) was analyzed (Section 2.5). For each time point a separate Eppendorf tube was incubated.

### 2.5. Aqueous Alcalase activity

The aqueous activity of Alcalase was assayed by monitoring the hydrolysis of 25% (v/v) ethyl lactate at 40 °C and pH 6.8 (10 ml of 100 mM sodium phosphate buffer pH 6.8, 20 ml of Milli-Q, and 10 ml of ethyl lactate). The resulting lactic acid was titrated with 0.1 mol $l^{-1}$ sodium hydroxide using pH-stat equipment (719 Stat Titrino Metrohm; Herisau, Switzerland). The pH-stat equipment was connected to a computer that logged the consumption of sodium hydroxide every 2 s. The method was based on a protocol obtained from ChiralVision [20]. The blank consumption of sodium hydroxide was monitored for 10 min. Subsequently the Alcalase formulation was added. The aqueous Alcalase activity is defined by the rate of sodium hydroxide consumption (corrected for the blank consumption of sodium hydroxide). The rate was based on 100 data points, thus in an interval of 200 s in total.

### 2.6. Settling rate

To get a rough indication of the settling rate of Cov and of molecular sieve beads and powder, they were added separately to 10 ml of THF in a 10 ml test tube of 9 cm in height [21]. After addition of Cov (40 mg), molecular sieve beads (800 mg), or molecular sieve powder (800 mg) to the THF, the mixture was shaken and subsequently allowed to settle. The settling time was measured and from this the settling rate was calculated [21].

### 2.7. Effect of reuse on the aqueous activity of Cov

Hydrated Cov (40 mg) was incubated in 1 ml of anhydrous THF at 25 °C in 2 ml Eppendorf safe-lock tubes placed on a blood rotator spinning at 40 rpm. Hydrated Cov was obtained by washing with successively 1 ml of each Milli-Q, anhydrous *t*-BuOH, and anhydrous THF. After 24 h, Cov was rehydrated by washing with successively 1 ml of each Milli-Q, anhydrous *t*-BuOH, and anhydrous THF. After a different number of incubation/washing steps, with a total of 8 recycles, the aqueous activity of Cov was analyzed (Section 2.5). This was done *in duplo*. The above procedure was also executed for:

(1) Cov (re)hydrated with 1 ml of 50 mM Tris buffer (pH 8) containing 20 mM calcium chloride instead of Milli-Q, in order to investigate the possible reversible loss of essential calcium ions during washing.
(2) Cov washed with Milli-Q, anhydrous *t*-BuOH, and THF, but after the washing step with Milli-Q, Cov was centrifuged for 10 instead of 2 min at 10,000 rpm, in order to further facilitate the separation of Milli-Q and Cov.
(3) Cov washed with Milli-Q and two times 1 ml of anhydrous THF. The washing step with anhydrous *t*-BuOH was thus omitted.
(4) Cov washed with two times 1 ml of anhydrous THF. The washing steps with Milli-Q and anhydrous *t*-BuOH were thus both omitted.