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Performance of Alcalase formulations in near dry organic media: Effect of enzyme hydration on dipeptide synthesis

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ABSTRACT

The use of different Alcalase formulations for protease-catalyzed dipeptide synthesis was investigated by studying the coupling of the carbamoylmethyl ester of N-protected phenylalanine with phenylalanine amide in tetrahydrofuran in the presence of molecular sieves (*i.e.* under near dry conditions). Hydration prior to drying (with anhydrous *tert*-butanol and anhydrous tetrahydrofuran) of the Alcalase formulations resulted in a significant increase in rate of the subsequent dipeptide synthesis. Repeated use, in the presence of molecular sieves, without intermediate rehydration led to inactivation of the enzyme. For three enzyme formulations this inactivation could be counteracted by intermediate rehydration. Inactivation of another enzyme formulation was only partially reversible by hydration. Alcalase immobilized onto dicalite with glutaraldehyde was found to be the most active in dipeptide synthesis, *i.e.* the formulation that initially produces the largest amount of product per gram of total formulation per unit of time. Due to its small particle size and its lack of operational stability, this formulation may nevertheless not be the best choice for the synthesis of dipeptides in neat organic media on a large scale. The most promising enzyme formulation for this is Alcalase covalently immobilized onto macroporous acrylic beads due to its reasonable activity, its seemingly good operational stability, and its size and uniform shape.

1. Introduction

Natural and non-natural peptides play an important role in the fields of health care, nutrition and cosmetics [1–5]. These peptides may be synthesized chemo-enzymatically for instance by protease-catalyzed coupling of an amino acid amide and a chemically synthesized C-terminally activated N-protected amino acid, *e.g.* an N-protected carbamoylmethyl (Cam) ester [6–11]. To prevent hydrolytic side reactions (hydrolysis of the activated amino acid, of the formed dipeptide, and of the protease itself), the availability of water in the system should be minimized, *e.g.* by executing the enzymatic peptide synthesis step in a neat organic solvent [12,13]. The system (reaction mixture and enzyme) should, however, not be completely dry as enzymes need some water to maintain their catalytically active conformation [10,12–21].

The present study focuses on the coupling of the carbamoylmethyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam) and phenylalanine amide (H-Phe-NH_2) (Fig. 1). A carbamoylmethyl ester was used instead of the more regularly used methyl or ethyl esters because of its positive effect on the coupling rate [7]. The coupling was executed with different commercially available Alcalase formulations (Alcalase is also referred to as subtilisin A or subtilisin Carlsberg [22–25]) in tetrahydrofuran (THF) in the presence of molecular sieves (*i.e.* under near dry conditions at low water activity), which were assumed to be stable in the process. In previous work we found that the rate of dipeptide synthesis could not be increased by increasing water activity values without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis/hydrolysis (S/H) ratio [10]. An appropriate amount of molecular sieves effectively prevents hydrolysis but still allows enzymatic activity [10].

Protease-catalyzed peptide synthesis in monophasic organic solvents has been studied previously, with different proteases: chymotrypsin [14,26–33], subtilisin Carlsberg [7,28,34–36], subtilisin BPN' [26], subtilisin 72 [37], thermolysin [31,38–40], Alcalase [6,41–43], and papain [44]. Nevertheless, the present contribution is to our knowledge the first to investigate the coupling of Z-Phe-OCam and Phe-NH_2 in THF with different Alcalase formulations ('hydrated' and 'dry') in the presence of molecular sieves (*i.e.* under near dry conditions at low water activity) and subsequently compare the formulations with respect to their initial peptide synthesis activity and their applicability on a large scale.

The aim of this study was twofold: (1) to investigate the effect of initial hydration state of selected Alcalase formulations, achieved

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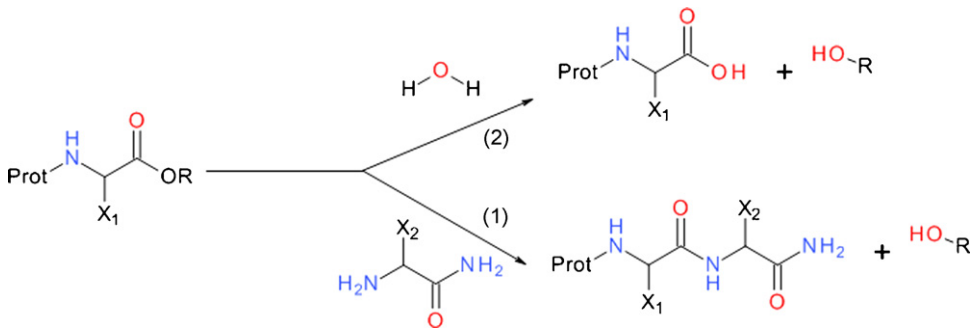

Fig. 1. Coupling of an N-protected, C-terminally activated amino acid with an amino acid amide to give a dipeptide (1), and hydrolysis of an N-protected, C-terminally activated amino acid (2). X_1 and X_2 can be any amino acid side chain, but are in this study both a benzyl side chain. R is an activating group. In this study R is carbamoylmethyl.

by a well-defined pretreatment protocol, on the subsequent dipeptide synthesis rate, and (2) to compare these formulations with respect to their applicability in dipeptide synthesis.

To achieve these aims, several commercially available Alcalase formulations, both 'hydrated' and 'dry,' were applied in the coupling of Z-Phe-OCam and Phe-NH_2. In addition, the repeated use (with and without intermediate rehydration) and thereby the operational stability of the four most active Alcalase formulations were compared. For this, the conversion of Z-Phe-OCam to dipeptide was monitored in time in two subsequent batch reactions. Furthermore, Alcalase formulation properties (particle size and shape, enzyme leaching from the support, and cost of immobilization) that are valuable for the synthesis of dipeptides in organic media were evaluated.

2. Materials and methods

2.1. Enzymes

The Alcalase formulations used in the present investigation and a description of the formulations are listed in Table 1.

The formulations from CLEA Technologies [45], ChiralVision [46], and DSM contain Alcalase from Novozyme Corp. The formulations from SPRIN Technologies contain Subtilisin A from *Bacillus species*. In the formulation from ChiralVision, Alcalase is covalently immobilized onto Immobeads 150 (cross-linked copolymer of methacrylate carrying oxirane groups) produced by ChiralVision in cooperation with FermentaBiotech Ltd. (Maharashtra, India).

2.2. Chemicals

tert-Butanol (*t*-BuOH) and THF were dried over activated 3 Å molecular sieves, 8–12 mesh beads ($K_nNa_{12-n}[(AlO_2)_{12}(SiO_2)_{12}]\cdot xH_2O$, Sigma Aldrich, 208582), prior to use. *t*-BuOH was melted by heating to 40 °C prior to use. The N-protected Cam-ester of phenylalanine (Z-Phe-OCam) and N-protected phenylalanyl-phenylalanine amide (Z-Phe-Phe-NH_2) were a kind gift from DSM (Geleen, The Netherlands), and used as supplied. Phenylalanine amide (H-Phe-NH_2) and N-protected phenylalanine (Z-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany). Deionized water (Milli-Q) was used throughout.

2.3. Enzyme pretreatment

The Alcalase formulations were pretreated in two different ways (the amounts of enzyme used are specified below, in Section 2.4). 'Dry' enzyme was obtained by washing with successively 1 ml of anhydrous *t*-BuOH and 1 ml of anhydrous THF. 'Hydrated' enzyme was obtained by washing with successively 1 ml of Milli-Q, 1 ml of anhydrous *t*-BuOH, and 1 ml of anhydrous THF. This treatment is expected to hydrate the enzyme, and to remove the excess water. A washing step involved adding washing liquid (Milli-Q, *t*-BuOH, or THF) to the Alcalase formulation, 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 with a pipette. This method was analogous to the procedure used to produce propanol-rinsed enzyme preparations [47,48].

Native Alcalase was not pretreated when applied 'dry,' assuming the freeze-dried powder to be dry. When native Alcalase was applied 'hydrated', it was hydrated by water vapor through the gas phase: it was placed for 96 h at 4 °C in an exsiccator containing Milli-Q.

2.4. Enzymatic peptide synthesis (1-timepoint measurement to compare Alcalase formulations)

For the enzymatic peptide synthesis (1-timepoint measurement), 150 μl THF containing 5.9 μmol H-Phe-NH_2 and 150 μl THF containing 3.5 μmol Z-Phe-OCam was added to the enzyme formulation. The amount of enzyme formulation was chosen to reach approximately 50% conversion of the Cam-ester in 1 h, with a 'hydrated' enzyme formulation. The following amounts of enzyme formulation were used: 5.5 mg of native Alcalase, 3 mg of Cov, 2.6 mg of CLEA-OM, 2.6 mg of CLEA-*t*Bu, 8.5 mg of CLEA-ST, 1 mg of Dic, 30 mg of S-epo, and 3 mg of S-imi. To obtain near dry conditions during peptide synthesis, 75 mg of 3 Å molecular sieves, 8–12 mesh beads, were added. Samples (50 μl) were diluted with 700 μl dimethyl sulfoxide (DMSO) to stop the reaction before HPLC analysis. The peptide synthesis was repeated 3 times for each enzyme formulation and carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed in a blood rotator spinning at 30 rpm.

The reaction was stopped after 1 h of incubation except for the conversions catalyzed by 'dry' Cov, 'dry' C-OM, and 'dry' S-epo, and native Alcalase. After 1 h of incubation, these formulations only achieved minimal conversion. For practical reasons (preventing the use of large amounts of enzyme), these formulations were incubated for 24 h in order to achieve a better measurable conversion.

2.5. Enzymatic peptide synthesis (repeated use)

To investigate the repeated use of the Alcalase formulations, 900 μl THF containing 29.4 μmol H-Phe-NH_2 and 900 μl THF containing 18.0 μmol Z-Phe-OCam were added to a 'hydrated' enzyme formulation. The peptide synthesis was carried out at 25 °C in 2 ml Eppendorf safe-lock tubes placed in a blood rotator spinning at 30 rpm. The enzyme concentrations were identical to the concentrations for the 1-timepoint measurements. To obtain near dry conditions, 10 mg of 3 Å molecular sieves (8–12 mesh beads) per mg of enzyme formulation were added. Samples (30 μl), that were

Table 1
Alcalase formulations: abbreviations as used in the present investigation, full name/description, particle diameter, and supplier.

Abbreviation	Full name/description	Particle diameter	Supplier
Native	Lyophilized native protease from *Bacillus licheniformis*	Fine powder	Sigma–Aldrich (Zwijndrecht, The Netherlands)
Cov	Alcalase covalently immobilized onto macroporous acrylic beads (Immobeads 150)	150–300 μm [62]	ChiralVision (Leiden, The Netherlands)
C-OM	Cross-linked enzyme aggregates (CLEA) containing Alcalase, CLEA-OM, formulation optimized for use in organic media	1 μm, but forms clusters [63]	CLEA Technologies (Delft, The Netherlands)
C-*t*Bu	Alcalase CLEA-*t*Bu, formulation precipitated and cross-linked in *tert*-butanol	1 μm, but forms clusters [63]	CLEA Technologies (Delft, The Netherlands)
C-ST	Alcalase CLEA-ST, standard CLEA formulation	1 μm, but forms clusters [63]	CLEA Technologies (Delft, The Netherlands)
Dic	Alcalase adsorbed onto dicalite 478 and cross-linked with glutaraldehyde	8 μm [64]	A kind gift from DSM (Geleen, The Netherlands)
S-epo	Alcalase covalently immobilized onto epoxy acrylic resin	100–300 μm [61]	SPRIN Technologies (Trieste, Italy)
S-imi	Alcalase covalently immobilized onto amino acrylic resin	100–300 μm [61]	SPRIN Technologies (Trieste, Italy)

taken in time, were diluted with 450 μl dimethyl sulfoxide (DMSO) before HPLC analysis.

After 24 h the Alcalase formulations were recycled. In the recycling procedure, the Alcalase formulation was first recovered from the reaction liquid. Then, fresh substrates were either added immediately to the Alcalase formulation, or the Alcalase formulation, from which the molecular sieve beads were separated one by one using tweezers, was first rehydrated by washing with successively 1 ml of each Milli-Q, anhydrous *t*-BuOH, and anhydrous THF, before adding fresh substrates and molecular sieve beads.

2.6. HPLC analysis

The amounts of dipeptide (Z-Phe-Phe-NH_2), Cam-ester (Z-Phe-OCam), and amino acid (Z-Phe-OH) were analyzed by HPLC (Thermo Separation Products P4000 pump and AS3000 autosampler) using a reversed-phase column (Inertsil ODS-3, C18, 5 μm, 150 mm × 4.6 mm) at 40 °C. UV detection was performed at 220 nm using an Ultimate 3000 Diode Array Detector (Dionex). The gradient program was: 0–25 min linear gradient ramp from 5% to 98% eluent B, 25–29 min linear gradient ramp back to 5% eluent B, 29–40 min 5% eluent B (eluent A: 0.5 ml l^{-1} methane sulfonic acid (MSA) in Milli-Q, eluent B: 0.5 ml l^{-1} MSA in acetonitrile). The flow was 1 ml min^{-1}. Injection volumes were 20 μl. Quantitative analysis was carried out using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

2.7. Aqueous Alcalase activity

The aqueous activity of the Alcalase formulations was determined 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 formed 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 [49]. The blanc consumption of sodium hydroxide was monitored for 10 min. Subsequently the Alcalase formulation was added and the consumption of sodium hydroxide was monitored for another 30 min. The Alcalase activity is defined by the rate of sodium hydroxide consumption (corrected for the blanc consumption of sodium hydroxide). The rate was determined after equilibration and was based on 100 data points in an interval of 200 s in total.

2.8. Scanning electron microscopy

The Alcalase formulations were placed onto aluminum holders with double-sided sticky carbon tape (EMS, Washington, USA). The samples were sputtered with platinum (JEOL, JFC 1200) and subsequently analyzed with a high resolution scanning electron microscope (FEI, Magellan 400) at room temperature at a working distance of 4.1–10.8 mm, with SE detection at 3.5 and 5 kV. Images were optimized by Photoshop CS5.

3. Results and discussion

3.1. Effect of the initial enzyme hydration state on dipeptide synthesis

The coupling of Z-Phe-OCam and H-Phe-NH_2 was performed with eight different Alcalase formulations (Table 1). The Alcalase formulations were either applied 'dry' (pre-treated with anhydrous *t*-BuOH and THF), or 'hydrated' (pre-treated with Milli-Q, anhydrous *t*-BuOH, and THF).

The effects of the initial enzyme hydration state on Z-Phe-OCam conversion (Fig. 2) were studied. Comparing Cam-ester conversion with and without initial enzyme hydration, it can be concluded that initial enzyme hydration causes a significant increase (*i.e.* no overlap in error bars) in the rates of Z-Phe-OCam conversion in case of Cov, C-OM, C-ST, Dic, S-epo, and S-imi (Fig. 2). Without initial hydration by Milli-Q washing, these enzymes seem to lack the minimal amount of water needed to maintain their catalytically active conformation [10,12–21]. This lack of water and therefore lack of enzyme activity is especially significant for Cov and C-OM. Initial hydration of C-*t*Bu does not cause an increase in the rate of Z-Phe-OCam conversion. Apparently, the crude untreated C-*t*Bu formulation was already sufficiently hydrated.

Only with 'hydrated' C-ST and S-epo, Z-Phe-OCam hydrolysis was observed (in the present system only the hydrolysis of substrate, and not of the product, occurs [10]), albeit minimal. Due to the low activity of C-ST and S-epo, a large amount of these enzyme formulations had to be added in order to achieve around 50% conversion of the Cam-ester in 1 h. The ratio of molecular sieves to enzyme formulation was therefore the lowest for C-ST and S-epo (respectively 9 and 2.5 mg molecular sieve beads per mg of enzyme formulation). Apparently, the amount of molecular sieves present was not sufficient to adsorb all the water left after washing the enzyme formulations with anhydrous *t*-BuOH and THF, and thus to prevent Cam-ester hydrolysis. For practical reasons (preventing the use of large amounts of molecular sieves) the amount of molecular sieves was not adjusted. With the other enzyme formulations, the Cam-ester was exclusively converted to dipeptide without

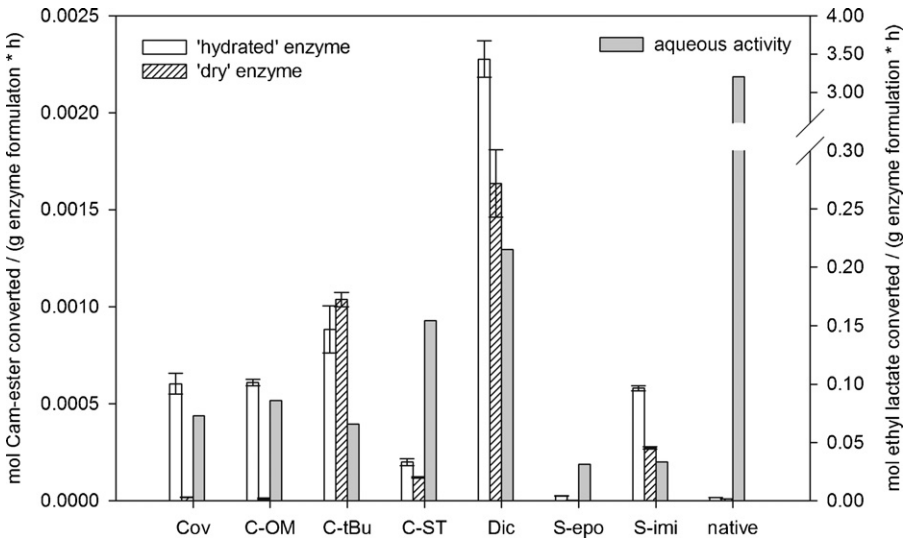

Fig. 2. Effect of initial hydration state of different Alcalase formulations on the conversion rate of Z-Phe-OCam (left *y*-axis) and the aqueous activity of the Alcalase formulations (right *y*-axis). The amount of converted Cam-ester was measured after 1 h of incubation at 25 °C, except for 'dry' Cov, 'dry' C-OM, 'dry' S-epo, and native Alcalase. These formulations were incubated for 24 h. The error bars are equal to the standard deviation of 3 independent measurements.

hydrolysis. In these cases, the amount of molecular sieve beads (>14 mg per mg of enzyme formulation) was apparently high enough to prevent Z-Phe-OCam hydrolysis.

3.2. Comparison of Alcalase formulations

In terms of Cam-ester conversion after 1 h of incubation, Dic, both in 'dry' and 'hydrated' form, is the most active enzyme formulation (Fig. 2), followed by C-*t*Bu (independent of the hydration state), 'hydrated' Cov, 'hydrated' C-OM, and 'hydrated' S-imi.

The least active enzyme formulations for the synthesis of Z-Phe-Phe-NH_2 are native Alcalase, C-ST, and S-epo (Fig. 2). Although hydration of native Alcalase, by water vapor through the gas phase, increased its synthetic activity, it was probably still too dry for significant enzymatic peptide synthesis in THF. The reason for the low activity of C-ST and S-epo is not clear, especially because the aqueous activity (*i.e.* the hydrolysis of ethyl lactate) of C-ST is 1.7 times higher than the activity of C-OM; the aqueous activities of S-epo and S-imi are similar (Fig. 2). One might think that the time for the hydration step (10 s) may not have been sufficient to hydrate C-ST and S-epo prior to use in organic solvent. We therefore washed C-ST en S-epo for 30 min with Milli-Q. The longer hydration step did, however, not remedy the low synthetic activity of C-ST and S-epo in organic solvent at all.

3.3. Repeated use of Alcalase formulations

For an Alcalase formulation to be used for dipeptide synthesis in organic media for longer periods of time, the operational stability of the enzyme formulation is important. We therefore studied the repeated use, with and without intermediate rehydration, of the four most active Alcalase formulations, *i.e.* 'hydrated' Cov, C-OM, C-*t*Bu, and Dic (Fig. 3A–D). Z-Phe-OCam was converted only to Z-Phe-Phe-NH_2 (thus was not hydrolyzed) for all 4 enzyme formulations. In the current system, data points requiring extended incubation and handling may have been affected somewhat by a certain amount of solvent evaporation (*e.g.* the concentration of Z-Phe-Phe-NH_2 after about 24 h of incubation is seen to be higher than the initial concentration of Z-Phe-OCam; Fig. 3A–D).

Without intermediate rehydration, the rate of Z-Phe-OCam conversion in the second batch was significantly lower than in the first one, for all four Alcalase formulations. This activity loss in time may either be reversible or irreversible. A possible cause for reversible inactivation is dehydration [50]: enzymes are known to require some essential water to maintain their catalytically active conformation [10,12–21]. Because molecular sieves remove water very efficiently, the enzyme formulation may slowly lose essential water and thus activity, as water is transferred from the enzyme to the molecular sieves. Indeed, in previous work, C-OM that had been pre-incubated with molecular sieve powder for 24 h before addition of substrates, showed minimal activity [10]. If such inactivation by dehydration is reversible, one should be able to restore activity by rehydrating the enzyme formulations before proceeding with the next batch.

Indeed, a nearly full reversal of inactivation was observed for Cov (Fig. 3A), C-OM (Fig. 3B), and C-*t*Bu (Fig. 3C). With intermediate rehydration, comparable activities were achieved for Cov, C-OM, and C-*t*Bu, in the first and second batch. Apparently, the activity loss observed without intermediate rehydration for Cov, C-OM, and C-*t*Bu, is caused by dehydration.

For Dic (Fig. 3D), the inactivation could not be completely reversed by intermediate rehydration. With intermediate rehydration, the activity was higher than without, but still significantly lower than the initial activity in the first batch. Apparently, Dic is partially irreversibly inactivated in THF in the presence of molecular sieves. Also, some Dic may have been lost during the washing steps.

It should be realized that, initially, the 'hydrated' enzyme formulations are clearly not at equilibrium with the dry solvent containing molecular sieves. During the incubation with the substrates and molecular sieves, the system will therefore change and move towards equilibrium, which involves dehydration of the enzyme formulation and thereby loss of enzymatic activity.

The kinetics of de- and re-hydration of the Alcalase formulations as well as the kinetics of the dipeptide synthesis will be studied in more detail in future work.

3.4. Evaluation of Alcalase formulations

From the above results it is concluded that initial hydration of Cov, C-OM, C-ST, Dic, S-epo, and S-imi, significantly increases the subsequent rate of Z-Phe-OCam conversion to Z-Phe-Phe-NH_2. In addition, Alcalase immobilized onto dicalite with glutaraldehyde (Dic) is the most active enzyme formulation for dipeptide synthesis

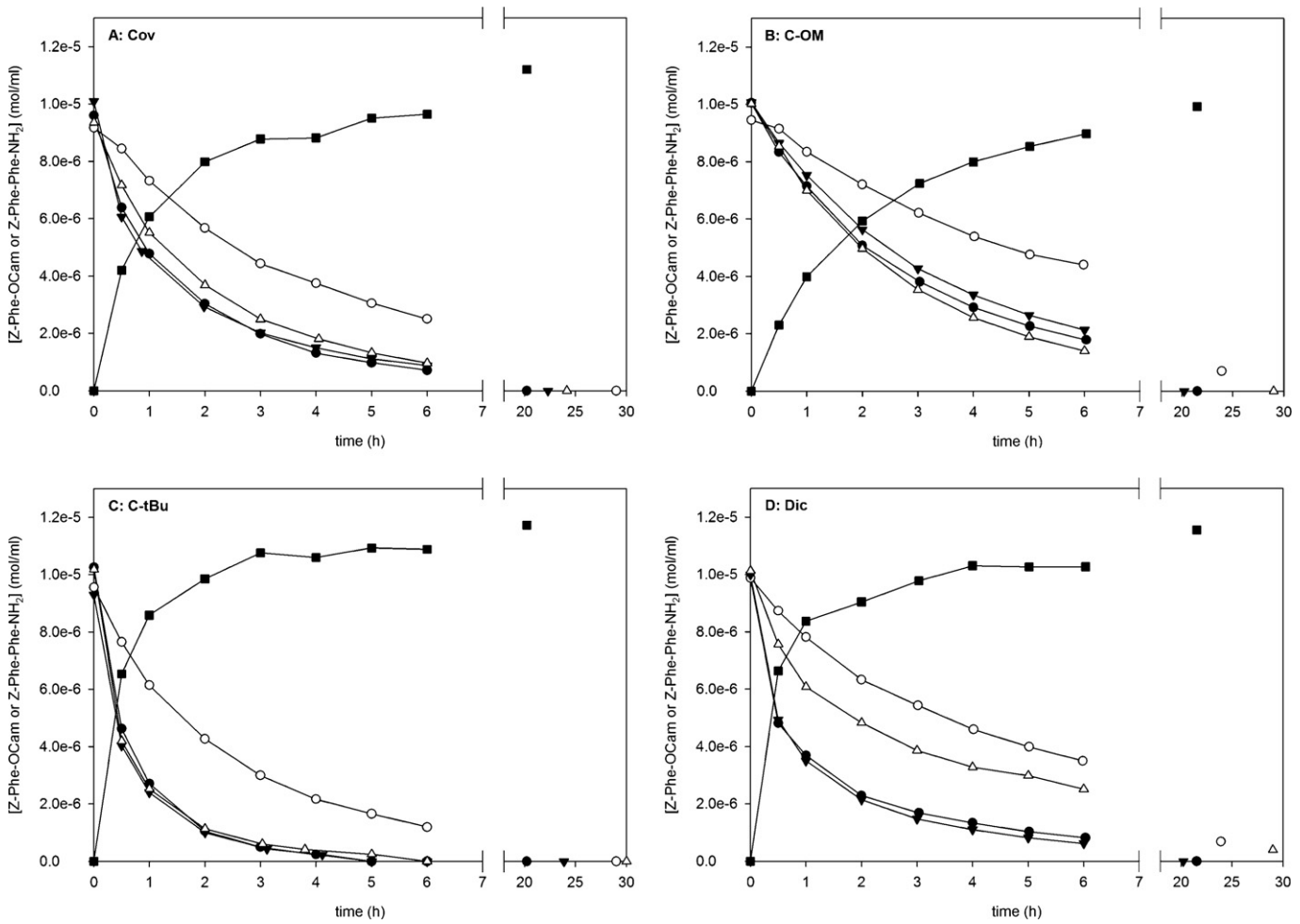

Fig. 3. Effect of repeated use, with and without intermediate rehydration, of Cov (A), C-OM (B), C-*t*Bu (C), and Dic (D), on the conversion of Z-Phe-OCam. Conversion of Z-Phe-OCam (●, ▼) and, for illustration, formation of Z-Phe-Phe-NH_2 (■) with an initially 'hydrated' enzyme formulation; subsequent conversion of Z-Phe-OCam, with (△) and without (○) intermediate rehydration.

(*i.e.* the formulation that initially produces the largest amount of product per gram of total formulation per unit of time).

Although Dic is most active, there are other properties to be considered when selecting an appropriate Alcalase formulation for the synthesis of dipeptides in neat organic media, such as particle size and shape, leaching of enzyme from the support, cost of immobilization method, enzyme loading, and operational stability [51,52]. We discuss each of these properties.

An enzyme formulation with a particle size exceeding 100–300 μm [52–54] can be easily separated from the reaction mixture, which is important when recycling and reusing a costly enzyme formulation and when enzyme contamination of the product should be minimized [53–58]. Apart from native Alcalase, Dic is the finest enzyme formulation (Table 1, Fig. 4). Its small size hampers separation. Separation of Dic from the reaction mixture will therefore be costly (*e.g.* centrifugation). The economic advantage of the good activity of Dic should thus be weighed against its poor separability. Filtration with common sieve plates will work for Cov, S-epo, and S-imi (Table 1, Fig. 4). The economic disadvantage of a lower activity of Cov and S-imi, compared to Dic may thus be compensated by their easier separation.

The particle shape of the enzyme formulation is important when the use of a packed-bed bioreactor is desired. The best candidates for the formation of a bed with good and stable flow properties are rigid and uniform particles that exceed 100–200 μm [58]. Small, deformable, and irregularly shaped particles can result in channeling, large pressure drops, and clogging [57–59]. Cov, S-epo, and S-imi, are the most promising formulations to be used in a packed-bed bioreactor due to their uniformity in shape and size (Fig. 4).

Enzyme leaching from a support may occur when an enzyme is physically adsorbed onto a carrier [53,60]. Leaching is a minor problem in organic solvents in which the enzymes are insoluble [52,53]. Hydration of the enzyme, however, may aggravate leaching. Dic is prepared by physical adsorbtion of Alcalase onto a carrier and then cross-linking it with glutaraldehyde. Cross-linked enzyme molecules may be trapped inside the pores of the dicalite, and leaching may be modest but not entirely suppressed. Leaching should not occur with Cov, the CLEAs, S-epo, and S-imi, as these enzyme formulations are covalently bound.

The costs of immobilization increase when a carrier is involved [55]. Therefore, the CLEAs are expected to have lower production costs on industrial scale than Cov, Dic, S-epo, and S-imi. On a small scale, the selling price of the CLEAs, (€ 225 for 5 g; information obtained from supplier) is, however, the highest compared to Cov (€ 99 for 5 g [46]), S-epo and S-imi (€ 25 for 5 g [61]).

The costs of an immobilized enzyme formulation increases if a large amount of native enzyme is needed to achieve a certain specific activity of the formulation. The exact enzyme loading can only be quantified during the immobilization procedure, and is unknown for the immobilized Alcalase formulations used in this investigation.

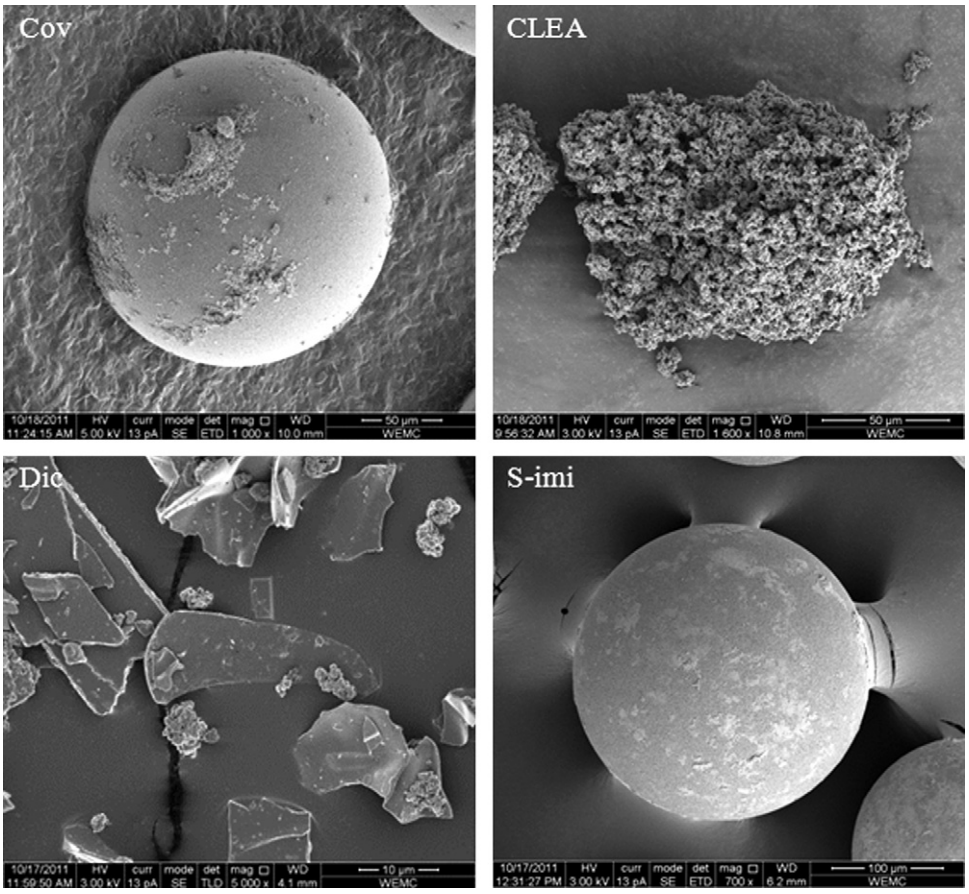

Fig. 4. Scanning electron microscope images of a Cov bead, a CLEA, Dic particles, and an S-imi bead (an S-epo bead looks identical to an S-imi-bead).

Operational stability of the enzyme formulation in organic solvents is essential when using an enzyme formulation for longer periods of time. Preferably, the activity of the enzyme formulation should not change significantly. If the activity does change, the enzyme formulation needs to be continuously replenished. In this work, the issue of operational stability was investigated for Cov, C-OM, C-*t*Bu, and Dic. When reusing the enzyme formulations, Cov, C-OM, and C-*t*Bu, could reach similar activities in two subsequent batch reactions if subjected to intermediate rehydration. In contrast, Dic is either partially irreversibly inactivated in THF in the presence of molecular sieves or is lost during the washing steps.

To conclude, although Dic is the most active enzyme formulation, it may not be the best choice for dipeptide synthesis in neat organic media on a large scale, due to its small size, cost of immobilization, and lack of operational stability. Cov, C-OM, and C-*t*Bu, may be better choices due to their good operational stability and covalent binding. For application in a packed-bed bioreactor, Cov may be the best choice due to the reasonable size and uniform shape. Therefore, Cov will be the focus of our future work on the kinetics of de- and re-hydration as well as the kinetics of the dipeptide synthesis.

4. Conclusions

Initial hydration of Alcalase covalently immobilized onto macroporous acrylic beads (Cov), Alcalase CLEA-OM (C-OM), Alcalase CLEA-ST (C-ST), Alcalase immobilized onto dicalite using glutaraldehyde (Dic), and Alcalase covalently immobilized onto acrylic beads (S-imi and S-epo) causes a significant increase in the rate of Z-Phe-Phe-NH_2 synthesis under dry conditions. Without such initial hydration, the enzymes seem to lack the water needed to maintain their catalytically active conformation.

When reusing Cov, CLEA-OM, CLEA-*t*Bu, and Dic, without intermediate rehydration, a significant decrease in the rate of dipeptide synthesis is observed. The activity loss observed without intermediate rehydration for Cov, C-OM, and C-*t*Bu, was caused by dehydration. Dic is either partially irreversibly inactivated in THF in the presence of molecular sieves or is lost during the washing steps.

Dic is the most active enzyme formulation (the formulation that initially produces the largest amount of product per gram of total formulation per unit of time) for the synthesis of Z-Phe-Phe-NH_2. Nevertheless, it may not be the best choice to synthesize dipeptides in neat organic media on a large scale mainly due to its small size and operational instability.

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