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Selecting optimal conditions for Alcalase CLEA-OM for synthesis of dipeptides in organic media

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ABSTRACT

In protease-catalyzed peptide synthesis, the availability of water is essential, as a compromise must be made between on the one hand the overall enzymatic activity and, on the other hand, the rate of product synthesis. Water is essential for enzyme activity, but at the same time causes hydrolytic side reactions. We studied the coupling of the carbamoylmethyl ester of N-protected phenylalanine and phenylalanine amide in tetrahydrofuran catalyzed by Alcalase CLEA-OM at a range of water activity (a_w) values, including the coupling in the presence of molecular sieves (*i.e.* at very low a_w values). The hydrolytic side reaction (in the present system only the hydrolysis of substrate occurs) was found to dominate above an a_w value of about 0.2. To prevent hydrolysis, the presence of molecular sieves was found to be necessary.

1. Introduction

Natural and non-natural peptides are important for pharmaceutical and food applications [1]. These peptides may be synthesized enzymatically. A possible peptide synthesis route, which is investigated in the frame of the Dutch national research program "Integration of Biosynthesis and Organic Synthesis (IBOS)," starts with activating the C-terminus of an N-protected amino acid using a lipase. Subsequently, this activated amino acid is coupled with an amino acid amide to give a dipeptide using a protease (Fig. 1, reaction 1) [2–5]. If 'free' water is present in the reaction medium, hydrolytic side reactions may proceed in addition to the coupling reaction (Fig. 1, reactions 2 and 3). To prevent the spontaneous hydrolysis of the activated amino acid (Fig. 1, reaction 2) as well as the enzyme-catalyzed hydrolysis of the activated amino acid, of the formed dipeptide (Fig. 1, reaction 3), and of the protease itself (autolysis), the availability of water in the system should be minimized. To achieve this, the coupling reaction can be carried out in a neat organic solvent. One should expect, however, that the organic solvent should not be completely dry as enzymes need some water to maintain their catalytically active conformation [6–8]. In a certain range, the more water, the greater the enzymatic activity [9]. Nevertheless, in the case of peptide synthesis (or any other reaction in which water is a substrate in hydrolytic reactions, e.g. transesterification) a compromise, with respect to the amount of water in the system, must be made between the overall enzymatic activity and the rate of specifically the product synthesis [10].

Thermodynamic water activity (a_w) is a measure of the availability of water in a system and reflects the extent of the interactions of water with other system components. It thus depends on the hydrophilic or hydrophobic nature of the solvent. To hydrophilic solvents a lot more water has to be added than to hydrophobic solvents to achieve the same a_w. The a_w value determines the hydration level of the enzyme and thereby also its activity [11,12].

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 (Phe-NH_2). A carbamoylmethyl ester was used instead of the more regularly used methyl or ethyl esters due to its positive effect on the coupling rate [13]. The coupling was catalyzed by Alcalase CLEA-OM in tetrahydrofuran (THF) at different a_w values. Protease-catalyzed peptide synthesis in monophasic organic solvents has been studied previously [4,14–17] including studies investigating the effect of water concentration on peptide synthesis catalyzed by different proteases: chymotrypsin [6,18–24], subtilisin Carlsberg [13,20,25,26], subtilisin BPN' [18], Alcalase [27–29], and papain [30]. Nevertheless, the present contribution is to our

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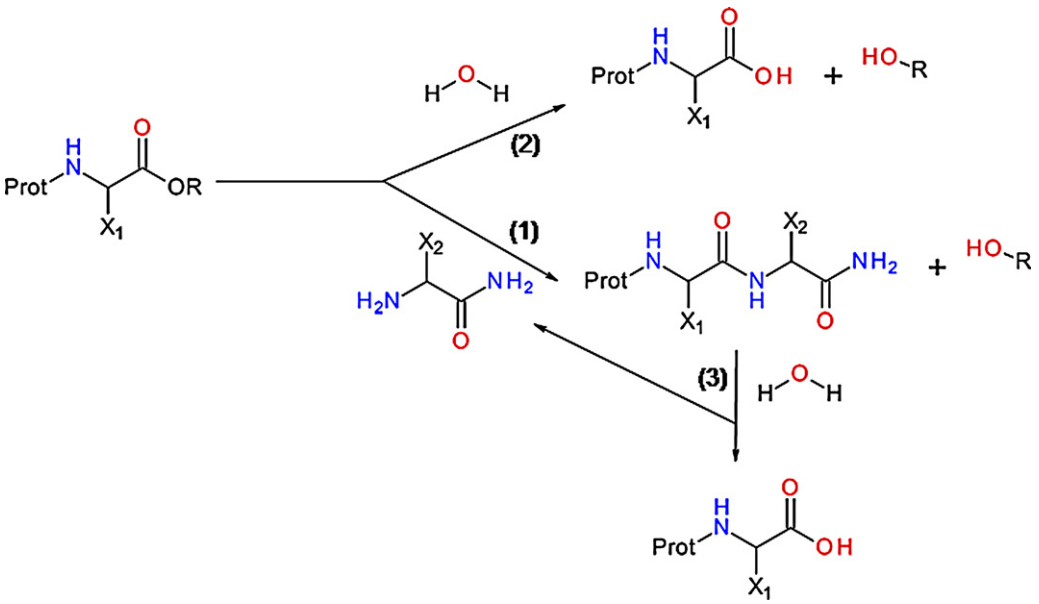

Fig. 1. Coupling of an N-protected, C-terminally activated amino acid with an amino acid amide to give a dipeptide (1), hydrolysis of an N-protected, C-terminally activated amino acid (2), and hydrolysis of the formed dipeptide (3). X_1 and X_2 can be any amino acid side chain, but are in this study both a benzyl side chain. *R* is an activating group. In this study *R* is carbamoylmethyl.

knowledge the first to investigate the coupling of Z-Phe-OCam and Phe-NH_2 in THF using Alcalase CLEA-OM at a range of a_w values, including the coupling in the presence of molecular sieves (*i.e.* at very low a_w values).

The aim of our study was to investigate up to which a_w value the rate of peptide synthesis could be increased without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis/hydrolysis (S/H) ratio. The a_w was varied from ~0 to 0.95 (in THF these a_w values correspond to 0–11%, v/v, water). In addition, the pH of the buffer used to wash Alcalase CLEA-OM was varied from 7 to 11 to examine whether this had an effect on subsequent coupling in near dry conditions. Furthermore, the pH of the buffer that was added directly to the reaction medium, to achieve an a_w of 0.6, was varied to observe whether this had an effect on the synthetic and hydrolytic reactions that take place at this a_w. The effect of the concentration of molecular sieves present in the reaction medium was studied in order to study the dipeptide synthesis at a_w values near 0.

2. Materials and methods

2.1. Enzymes

Alcalase® (protease from *Bacillus licheniformis*) CLEA-OM® (formulation optimized for use in organic media) was obtained from CLEA Technologies (Delft, The Netherlands). Alcalase CLEA-OM contains the enzyme Alcalase from Novozyme Corp.

2.2. Chemicals

All chemicals used were reagent or analytical grades. *tert*-Butanol (*t*-BuOH) and THF were dried over 3 Å molecular sieves, 8–12 mesh beads (Sigma–Aldrich), for at least 1 day prior to use. *t*-BuOH was pre-heated to a liquid (40 °C) prior to use. Z-Phe-OCam and Z-Phe-Phe-NH_2 were a kind gift from DSM (Geleen, The Netherlands). Phe-NH_2, N-protected phenylalanine (Z-Phe-OH), and N-protected phenylalanyl-phenylalanine (Z-Phe-Phe-OH) were purchased from Bachem (Weil am Rhein, Germany).

2.3. Setting water activity

The a_w was set by adding an appropriate amount of water to the THF, to which the enzyme and substrates were added. The amount of water that needed to be added to achieve a certain a_w was calculated using the activity coefficient (γ_w) and the mole fraction of water (x_w)

$$a_w = \gamma_w x_w \quad (1)$$

γ_w can be calculated from [31–33]:

$$\ln(\gamma_w) = -\ln(x_w + \Lambda_{ws}(1 - x_w)) + (1 - x_w)\left(\frac{\Lambda_{ws}}{x_w + \Lambda_{ws}(1 - x_w)} - \frac{\Lambda_{sw}}{\Lambda_{sw}x_w + (1 - x_w)}\right) \quad (2)$$

where

$$\Lambda_{ws} = \frac{V_s}{V_w}\exp\left(\frac{-(\lambda_{ws} - \lambda_{ww})}{RT}\right) \quad (3)$$

and

$$\Lambda_{sw} = \frac{V_w}{V_s}\exp\left(\frac{-(\lambda_{sw} - \lambda_{ss})}{RT}\right) \quad (4)$$

where V_s and V_w are the molar volumes of the organic solvent, in this case THF and water, respectively. V_s is 81.55 ml mol^{-1} at 25 °C and V_w 18.07 ml mol^{-1} [31]. For a THF-water mixture the value of the $\lambda_{ws} - \lambda_{ww}$ Wilson coefficient is 7603 J mol^{-1} and the value of the $\lambda_{sw} - \lambda_{ss}$ Wilson coefficient is 11,212 J mol^{-1} [31]. *R* is the gas constant (8.314 J mol^{-1} K^{-1}) and *T* the absolute temperature (K).

The above calculation is based on a binary system, in this case water and THF. For the enzymatic peptide synthesis the system contained additional compounds such as substrates, an enzyme formulation, and, in time, products. As the concentration of substrates (53 mM Phe-NH_2 and 37 mM Z-Phe-OCam) and the amount of enzyme added to the reaction mixture (6 mg ml^{-1}) is rather low, we assume the a_w of our system to be similar to the calculated a_w based on a binary system.

The water content of the THF solutions was measured by Karl–Fischer titration (Mettler Toledo DL38; Tiel, The Netherlands) after the incubation period, in which dipeptide synthesis could take

place. Based on this measured water content, the a_w of the reaction mixtures was recalculated using the equations above.

We chose to set the a_w by adding an appropriate amount of water to the THF instead of equilibrating with saturated salt solutions or salt hydrate pairs [34]. When THF was equilibrated with a saturated salt solution for 24 h, a lot of THF evaporated and was found in the salt solution. When solvents dissolute in the salt solution it can alter the a_w of the salt solution [35]. In addition, we did not want to add the salt hydrate pairs directly to the system containing the enzyme because the salt hydrates may not be compatible with the enzyme [35] and the salt hydrates may have acid–base effects on the enzyme [36].

2.4. Enzymatic peptide synthesis

Enzymatic reactions were carried out at 25 °C in 1.5 ml glass vials placed in a blood rotator spinning at 15 rpm. Alcalase CLEA-OM was washed successively with 1 ml of anhydrous *t*-BuOH and 1 ml of THF to remove residual water. This washing step involved adding the solvent to the Alcalase formulation, shaking the sample, allowing the sample to settle, and removing the solvent using a pipette. This method was analogous to the procedure used to produce propanol-rinsed enzyme preparations [37,38].

For some experiments, Alcalase CLEA-OM was washed with 1 ml of Milli-Q or buffer prior to washing with *t*-BuOH and THF.

In an attempt to achieve a completely dry Alcalase CLEA-OM formulation, 75 mg 3 Å molecular sieves, 8–12 mesh beads, were added to 1.7 mg of solid Alcalase CLEA-OM particles. The mixture was stored at 4 °C for 18 h prior to use.

In order to dry Alcalase CLEA-OM in the presence of THF, different amounts of 3 Å molecular sieve powder (Sigma–Aldrich) were added to the enzyme preparation that was incubated in THF at 25 °C for 24 h prior to use.

For the enzymatic peptide synthesis, 150 μl THF containing 16 μmol Phe-NH_2 and 150 μl THF containing 11 μmol Z-Phe-OCam were added to 1.7 mg of the enzyme. To set the a_w of the reaction mixture, either molecular sieves or an appropriate amount of water were added. Samples (50 μl) were diluted with 700 μl dimethyl sulfoxide (DMSO) before HPLC analysis.

Except for the reactions used to investigate dipeptide synthesis at a_w values near 0, 75 mg 3 Å molecular sieves, 8–12 mesh beads, were used. For the reactions used to investigate dipeptide synthesis at a_w values near 0, different amounts of 3 Å molecular sieve powder were added to the reaction mixture instead of the coarse beads. Due to the small volumes used in our system, the amount and therefore the adsorptive capacity of powder can be controlled better (by weighing off a specific amount of powder) than that of beads. We assume the molecular sieve powder to have the same adsorptive capacity for water molecules as the molecular sieve beads because, similarly to the beads, it was sold as a drying agent. Furthermore, the powder has the same elemental composition as the beads.

To study the effect of pH on dipeptide synthesis, the following buffers were used: 50 mM Tris (hydroxylmethyl) aminomethane – hydrochloric acid buffer (pH 7–9), 50 mM sodium carbonate – sodium bicarbonate buffer (pH 9.5–10.5), and 50 mM sodium bicarbonate buffer – sodium hydroxide (pH 11).

2.5. Substrate and dipeptide (product) hydrolysis

To investigate whether hydrolysis of the substrate, Z-Phe-OCam, takes place in our system, 300 μl THF containing 11 μmol Z-Phe-OCam was incubated at 25 °C with 1.7 mg of Alcalase CLEA-OM, that was not washed with anhydrous *t*-BuOH and THF prior to use. A sample, for HPLC analysis, was taken after 24 h of incubation time.

To test spontaneous substrate hydrolysis, 150 μl THF containing 15 μmol Phe-NH_2 and 150 μl THF containing 11 μmol Z-Phe-OCam were incubated at 25 °C without enzyme at a_w values ranging from 0.4 to 0.95. Samples, for HPLC analysis, were taken after 24 h, and after 6 or 8 days of incubation time.

To investigate whether hydrolysis of the dipeptide, Z-Phe-Phe-NH_2, takes place in our system, Z-Phe-Phe-OH was incubated at 25 °C with 1.7 mg of Alcalase CLEA-OM, that was washed successively with 1 ml anhydrous *t*-BuOH and THF prior to use, at a range of a_w values (a_w = 0–0.95). The possible hydrolysis of Z-Phe-Phe-OH was assumed to be similar to Z-Phe-Phe-NH_2. Samples, for HPLC analysis, were taken after 24 h and 7 days of incubation time.

2.6. HPLC analysis

The amounts of dipeptide (Z-Phe-Phe-NH_2), Z-Phe-OCam, and 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 H_2O, 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 performed using calibration curves of Z-Phe-Phe-NH_2, Z-Phe-OCam, and Z-Phe-OH.

HPLC analysis was not a problem for the system in which CLEA-OM and molecular sieve beads, or CLEA-OM and molecular sieve powder were used, as enough reaction liquid remained for the analysis.

3. Results and discussion

3.1. Substrate and dipeptide (product) hydrolysis

To investigate whether hydrolysis of the substrate, Z-Phe-OCam (Fig. 1, reaction 2), takes place in our system, Z-Phe-OCam was incubated with Alcalase CLEA-OM, that was not washed with anhydrous *t*-BuOH and THF prior to use. After 24 h of incubation in the absence of both molecular sieves and additional water, Z-Phe-OH was detected. The original Alcalase CLEA-OM formulation thus contains enough water for hydrolysis of Z-Phe-OCam to take place.

Z-Phe-OCam was also incubated without enzyme at a_w values ranging from 0.4 to 0.95, to test spontaneous hydrolysis. At a_w values ranging from 0.4 to 0.8 no Z-Phe-OH was detected after 6 days of incubation. At an a_w value of 0.95, 5% of the Z-Phe-OCam was hydrolyzed after 24 h and 35% after 8 days of incubation. So only above an a_w value of minimally 0.8 some measurable spontaneous hydrolysis of the substrate occurs.

To investigate whether hydrolysis of the dipeptide, Z-Phe-Phe-NH_2 (Fig. 1, reaction 3), takes place in our system, Z-Phe-Phe-OH was incubated with Alcalase CLEA-OM, that was washed successively with anhydrous *t*-BuOH and THF prior to use, at a range of a_w values (a_w = 0–0.95). The possible hydrolysis of Z-Phe-Phe-OH was assumed to be similar to Z-Phe-Phe-NH_2. After 7 days of incubation, no Z-Phe-OH was detected. Also in later reactions in which Z-Phe-Phe-NH_2 was formed, no product hydrolysis was apparent in time. Hydrolysis of the dipeptide (Fig. 1, reaction 3) thus does not take place in our system. This is in line with the work from Li et al. in which no hydrolysis of Z-Asp-Val-NH_2 was observed by native Alcalase in acetonitrile containing 10% buffer [28]. Also Hou et al. did not observe hydrolysis of Bz-Arg-Gly-NH_2 by Alcalase in acetonitrile containing 10% buffer [29]. Although Jönsson et al. did observe hydrolysis of Ac-Phe-Ala-NH_2 (by immobilized α-chymotrypsin in acetonitrile containing 10% water), the

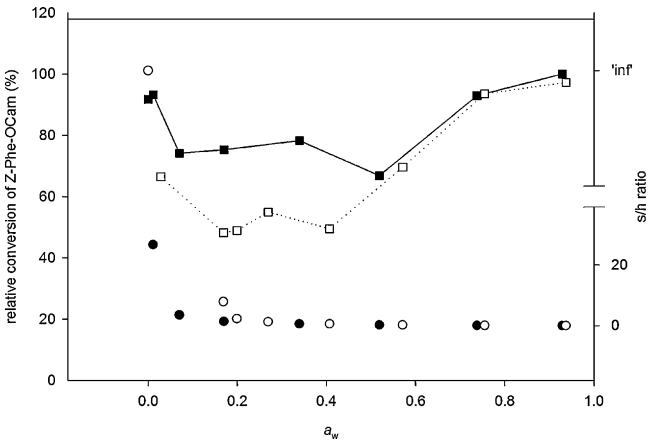

Fig. 2. Effect of a_w on the total catalytic activity (■) and on the synthesis/hydrolysis (S/H) ratio (●) of Alcalase CLEA-OM immediately, after washing with anhydrous *t*-BuOH and THF, added to the reaction mixture and on the total catalytic activity (□) and on the S/H ratio (○) of Alcalase CLEA-OM equilibrated (24 h) before addition to the reaction mixture. The total conversion of Z-Phe-OCam at a certain a_w is relative to the highest conversion obtained.

substrate ester, Ac-Phe-OEt, was a considerably better substrate for hydrolysis than the dipeptide [19].

Apparently, Z-Phe-OCam is a much better substrate in the hydrolytic reaction for the enzyme than the current dipeptide; Z-Phe-Phe-NH_2 hydrolysis can thus be neglected. In the present system the incubation time is thus not a critical parameter in the optimization of the dipeptide synthesis because once the dipeptide is formed, it will not be hydrolyzed.

3.2. Effect of a_w on dipeptide synthesis

The coupling of Z-Phe-OCam and Phe-NH_2, catalyzed by Alcalase CLEA-OM, was investigated at a range of a_w values. The enzyme formulation was washed successively with anhydrous *t*-BuOH and THF before the substrates, and molecular sieve beads or an appropriate amount of water were added. The enzyme and substrates were incubated for 24 h. In the presence of molecular sieves the a_w of the reaction mixture can be safely assumed to be the lowest attained and approaching zero, $a_w \sim 0$.

Fig. 2 shows the effect of a_w on the total catalytic activity (synthesis plus hydrolysis). The a_w value on the *x*-axis is a recalculated a_w value according to the THF water content determined with Karl–Fischer titration after peptide synthesis. The relative conversions in Fig. 2 (left *y*-axis) were calculated by normalization with respect to the conversion at $a_w = 0.95$ with CLEA-OM (washed with anhydrous *t*-BuOH and THF, immediately added to the reaction mixture). At an a_w value of 0.95, 6.6 mmol Z-Phe-OCam was converted per gram of CLEA-OM in 24 h. Next to achieving a high catalytic activity at high a_w values, a high catalytic activity was also achieved at very low a_w ($a_w < 0.1$) values. The amount of spontaneous hydrolysis of Z-Phe-OCam at high a_w ($a_w > 0.8$) values, mentioned above, was negligible compared to the total amount of hydrolysis at these a_w values.

Fig. 2 also shows the effect of a_w on the synthesis/hydrolysis (S/H) ratio. The hydrolysis reaction consists only of the hydrolysis of the activated amino acid (Fig. 1, reaction 2). At extremely low a_w values, the S/H ratio is theoretically infinite ('inf') as no hydrolysis of the substrate was measured. The S/H ratio decreases rapidly with increasing a_w values. Based on the measured S/H ratio values at different a_w values, it can be concluded that from an a_w value of about 0.2, the S/H ratio becomes less than 1. The hydrolysis reaction thus dominates above an a_w value of about 0.2. At an a_w value of 0.95, only a minimal amount of dipeptide (Z-Phe-Phe-NH_2) was synthesized. The S/H ratio at this a_w is 0.01.

If the hydrolysis of the substrate should be minimal because the substrate is, for example, very expensive, the a_w should thus be low ($a_w \ll 0.2$). To prevent the formation of a hydrolysis product (Z-Phe-OH), the presence of molecular sieves was found to be necessary, *i.e.* the a_w must be extremely low to prevent hydrolysis. In the absence of both molecular sieves and additional water, hydrolysis was still observed. This means that there is still enough water left on the enzyme formulation that can compete with the dipeptide synthesis reaction. The successive washing steps with anhydrous *t*-BuOH and THF thus do not remove all the water from the enzyme formulation. The loss of water from the enzyme formulation to the bulk solvent means that the two pertinent phases (*i.e.* the THF and the enzyme formulation) were not at equilibrium. The a_w of the bulk solvent was set by the addition of an appropriate amount of water to the THF. During the 24 h incubation period for peptide synthesis, the water was then likely redistributing between the CLEA-OM and the bulk solvent.

In order to compare the above results with a system in which CLEA-OM is equilibrated to the same a_w as the bulk solvent, the experimental set-up was modified. Before incubating the enzyme and substrates for 24 h, CLEA-OM was incubated with 1.5 ml of THF at a range of a_w values. The water transferred from or to the enzyme was so minimal that it did not measurably change the a_w of the bulk THF. After 24 h, the THF was removed and THF containing the substrates was added. The a_w of the THF containing the substrates was set 4 h before incubation with the enzyme. As was observed in the system in which CLEA-OM was used immediately, the highest catalytic activity was achieved at the highest a_w value tested (0.95) (Fig. 2). Nevertheless, in comparison to the system in which CLEA-OM was used immediately, a lower catalytic activity was achieved at a_w values between 0 and 0.4. The relative rate remained approximately constant at 52% in the a_w range of 0.2 to 0.4. Above an a_w value of 0.4, the relative rate increased with a_w. As was observed in the system in which CLEA-OM was used immediately, the S/H ratio in the equilibrated system also decreased rapidly with increasing a_w values and the hydrolysis reaction remained to dominate above an a_w value of about 0.2 (Fig. 2).

The main difference between the system in which CLEA-OM was used immediately and the equilibrated system, is the relatively high catalytic activities observed in the system in which CLEA-OM was used immediately at the lower a_w ($a_w < 0.4$) values. These high enzymatic activities, as compared to those observed with the equilibrated system, indicate that it might be quite interesting to use this 'hydration gradient' (and therefore non-equilibrium situation) between the enzyme formulation and the bulk solvent in dipeptide synthesis. Indeed, initially relatively high catalytic activities and high S/H ratios can be achieved using a relatively dry bulk solvent and a hydrated enzyme formulation. In line with this thought, Basso et al. developed an innovative method to maintain the hydration state of the enzyme while preventing hydrolytic side reactions. They adsorbed hydrated thermolysin onto Celite rods and performed an enzymatic dipeptide synthesis in toluene at an a_w value of 0.7–0.75 [39].

3.3. Effect of pH on dipeptide synthesis

The pH of the buffer used to wash Alcalase CLEA-OM was varied from 7 to 11 to examine whether this had an effect on the subsequent coupling in near dry conditions (Fig. 3). The following buffers were used: 50 mM Tris (hydroxylmethyl) aminomethane – hydrochloric acid buffer (pH 7–9), 50 mM sodium carbonate – sodium bicarbonate buffer (pH 9.5–10.5), and 50 mM sodium bicarbonate buffer – sodium hydroxide (pH 11). Miyazawa et al. observed a large effect of the pH of the buffer solution from which

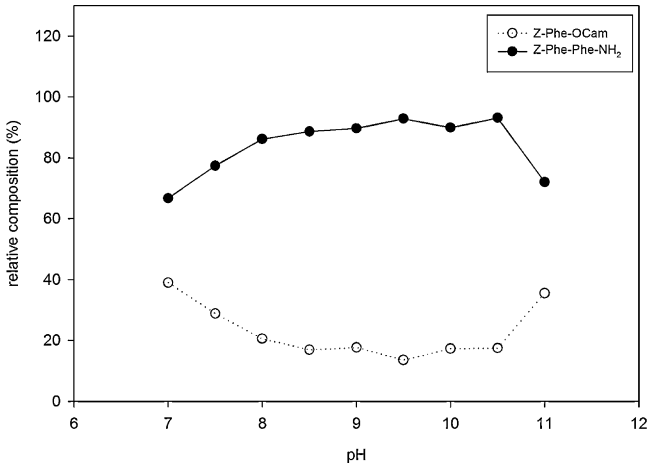

Fig. 3. Effect of the pH of the washing buffer solution on the relative composition of the reaction mixture after 24 h of incubation at 25 °C using Alcalase CLEA-OM.

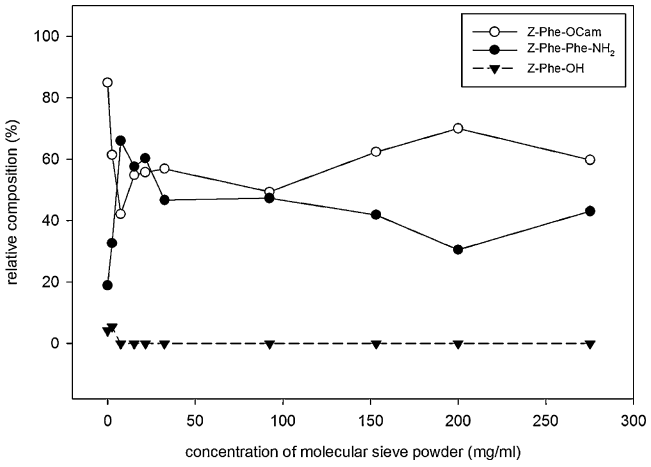

Fig. 5. Effect of different concentrations of molecular sieve powder on the relative composition of the reaction mixture after 3 h of incubation at 25 °C using Alcalase CLEA-OM.

lyophilized enzymes were prepared on the peptide yield, with an optimum at pH 10.7 [13]. In addition, Li et al. observed a significant effect of the pH of the 10% (v/v) buffer solution that was added to acetonitrile on the peptide yield, with an optimum at pH 10 [28].

Alcalase CLEA-OM was hydrated with buffer and subsequently washed with anhydrous *t*-BuOH and THF. The enzyme and substrates were incubated for 24 h in the presence of molecular sieve beads.

The *y*-axis of Fig. 3 (as well as the *y*-axis of Figs. 4 and 5) is labeled 'relative composition.' The relative composition of, for example, Z-Phe-Phe-NH_2 was calculated as follows:

$$\text{relative composition ZPhePheNH}_2\,(\%) = \frac{[\text{ZPhePheNH}_2]_t}{[\text{ZPheOCam}]_0} \times 100$$

where $[\text{ZPhePheNH}_2]_t$ is the concentration of Z-Phe-Phe-NH_2 at the time of measurement and $[\text{ZPheOCam}]_0$ is the initial concentration of Z-Phe-OCam, both in mol ml^{-1}.

The pH of the buffer used to wash Alcalase CLEA-OM did have an effect on the subsequent coupling (Fig. 3). A broad pH optimum exists ranging from pH 8.0 to 10.5. Likely the small amounts of buffer that were present in the reaction mixture and surrounding the enzyme have an effect on the synthetic activity of the enzyme. These residual amounts of buffer were, however, so small that no hydrolysis was observed. This enhancement in synthetic activity between pH 8.0 and 10.5 coincides partly with the pH optimum of Alcalase activity in aqueous conditions, which is between pH 8 and 9 [40]. In addition, only deprotonated Phe-NH_2 can react to form the dipeptide and this form of Phe-NH_2 is available at alkaline pH [7,41].

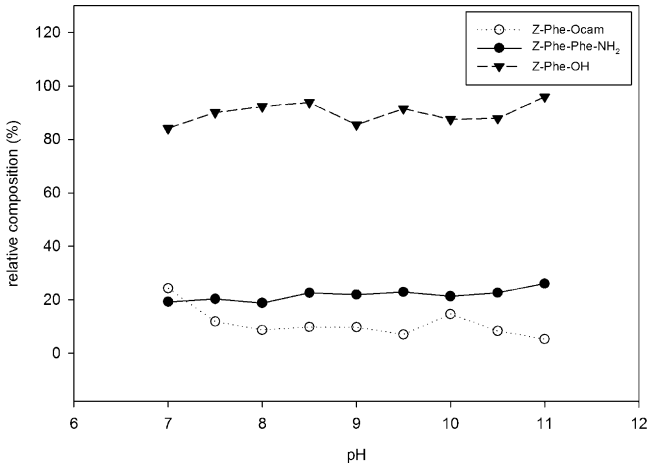

Fig. 4. Effect of the pH of the buffer solution that was added to achieve an a_w of 0.6 on the relative composition of the reaction mixture after 24 h of incubation at 25 °C using Alcalase CLEA-OM.

In order to investigate whether a pH optimum also exists when buffer is added directly to the reaction medium, the effect of the buffer pH on the reactions catalyzed by Alcalase CLEA-OM at an a_w of 0.6 was studied (Fig. 4). At an a_w of 0.6 both synthesis and hydrolysis reactions occur. Alcalase CLEA-OM was hydrated with buffer, that had the same pH and concentration as the buffer added to the reaction mixture, and subsequently washed with anhydrous *t*-BuOH and THF. The same buffers were used as above, when studying the effect of the washing buffer pH on the subsequent coupling in near dry conditions. The enzyme and substrates were incubated for 24 hours at an a_w of 0.6. To achieve an a_w of 0.6, 5.9 μl of buffer was added to 300 μl of THF containing 1.7 mg CLEA-OM.

In a monophasic organic solvent, pH is undefined [42]. The enzyme is a polyelectrolyte with a certain ionization state. The addition of 5.9 μl buffer to the reaction mixture (to achieve an a_w of 0.6) means that about 35 μg of buffer salts were added. It is doubtful whether this small amount of buffer salts can alter the ionization state of 1.7 mg CLEA-OM. The washing step with 1 ml of buffer before incubation, however, may have a more significant effect on the ionization state of the enzyme and therefore on the synthesis and hydrolysis reactions that occur (as was seen when studying the effect of the washing buffer pH on the subsequent coupling in near dry conditions), as the volume used is much larger.

The rates of dipeptide synthesis and substrate hydrolysis do not seem to be significantly affected by the pH of the buffer solution that was added to the reaction mixture (Fig. 4). No optimum was observed. The pH of the buffer at an a_w of 0.6 neither affects the synthetic nor the hydrolytic activity of the enzymes significantly at the optimum pH range.

3.4. Dipeptide synthesis at very low a_w values

As stated before, the a_w of the reaction system must be extremely low to prevent hydrolysis. Nevertheless, the system should not be completely dry as enzymes need some water to maintain their catalytically active conformation [6–8]. We believe that an optimum in solely dipeptide synthesis can be achieved when the dipeptide synthesis is carried out near an a_w value of the bulk solvent of 0. In order to achieve a_w values near 0, molecular sieve powder, of which the concentration was varied, instead of

molecular sieve beads were used. Due to the small volumes used in our system, the amount and therefore the adsorptive capacity of powder can be controlled better (by weighing off a specific amount of powder) than that of beads.

The effect of the concentration of molecular sieves present in the reaction medium on the coupling of Z-Phe-OCam and H-Phe-NH_2 was investigated (Fig. 5). Alcalase CLEA-OM was first hydrated with Milli-Q and subsequently washed with anhydrous *t*-BuOH and THF. The enzyme and substrates were incubated for 3 h in the presence of different amounts of molecular sieve powder.

With Karl–Fischer titration it was not possible to accurately determine the water content of these samples as the water content is too low. Therefore the specific a_w of the reaction systems containing different amounts of molecular sieve powder could not be calculated.

An optimum in dipeptide synthesis was achieved when the dipeptide synthesis was carried out in the presence of about 7.5–20 mg ml^{-1} of molecular sieve powder (Fig. 5). When only a small amount of molecular sieve powder (<3 mg ml^{-1}) was added to the reaction mixture hydrolysis could not be prevented. When a relatively large amount of molecular sieve powder was added to the reaction mixture (>35 mg ml^{-1}) the rate of dipeptide synthesis was significantly reduced. Apparently, at this point, the activity of Alcalase CLEA-OM was reduced due to dehydration.

The above investigation of the effect of the concentration of molecular sieves present in the reaction medium on the coupling of Z-Phe-OCam and H-Phe-NH_2 using Alcalase CLEA-OM was carried out in triplicate. Fig. 5 is an illustrative example of the three series done. The results all show the same trend.

If it is assumed that molecular sieves are a perfect sink or at least have a very high partition coefficient for water, the enzyme formulation and the solvent may lose water in time. This would lead to inactive enzyme formulations as enzymes are known to require some essential water to maintain their catalytic active conformation [6–8]. An attempt was made to dry the Alcalase CLEA-OM formulation even further than with anhydrous *t*-BuOH and THF alone by storing it with molecular sieve beads for 18 hours before use in an enzymatic reaction. Indeed, this extremely dry Alcalase CLEA-OM formulation was found totally incapable of dipeptide synthesis. To have an indication whether the enzyme formulation loses water in time in the presence of THF and molecular sieves, and thereby inactivates, non-washed Alcalase CLEA-OM was incubated in THF with different amounts of molecular sieve powder for 24 h before adding the substrates. The Alcalase CLEA-OM pre-incubated with 6-17 mg ml^{-1} molecular sieve powder converted on average 2.4% of the substrate to dipeptide and 0.9% to Z-Phe-OH in 3 h. The Alcalase CLEA-OM pre-incubated with 28–635 mg ml^{-1} molecular sieve powder converted on average 1.6% of the substrate to dipeptide in 3 h and no hydrolysis was observed. Pre-incubation with a small amount of molecular sieve powder thus caused a somewhat higher dipeptide synthesis rate but hydrolysis could not be prevented. In any case, the conversion achieved by the Alcalase CLEA-OM formulation pre-incubated with molecular sieve powder is significantly lower than the >40% conversion achieved by the Alcalase CLEA-OM formulation to which molecular sieve powder was added at the same time as the substrates (Fig. 5). The pre-incubation with molecular sieve powder thus seems to have dried the enzyme to such an extent that only a minimal activity remained. This result suggests that although the rate of catalysis in the systems to which molecular sieves are added at the same time as the substrates is very high in the first hours of incubation, due to the initially hydrated and thus active enzyme, the rate of catalysis will be minimal after 24 h of incubation, due to the significant dehydration of the enzyme by the molecular sieves. The exact rate of this dehydration and its effect on the enzyme activity are subject to further study.

4. Conclusions

In the present system the incubation time is not a critical parameter in the optimization of the dipeptide synthesis because once the dipeptide is formed, it will not be hydrolyzed. Only hydrolysis of the substrate, Z-Phe-OCam, takes place.

The rate of peptide synthesis could not be increased by increasing a_w values without significantly increasing the rate of hydrolysis, *i.e.* without significantly decreasing the synthesis/hydrolysis (S/H) ratio. At extremely low a_w values, *i.e.* in the presence of an excess amount of molecular sieves, the S/H ratio is theoretically infinite as no hydrolysis product was measured. Above an a_w value of about 0.2, the hydrolysis reaction dominates.

The pH of the buffer used to wash Alcalase CLEA-OM has an effect on the coupling. A broad pH optimum exists ranging from pH 8.0 to 10.5. The pH of the buffer that is added directly to the reaction medium, to achieve an a_w of 0.6, neither affects the synthetic nor the hydrolytic activity of the enzyme.

An appropriate amount of molecular sieves can prevent hydrolysis of the substrate and still allow enzymatic activity. Nevertheless, if Alcalase CLEA-OM is pre-incubated with molecular sieves for 24 h before adding substrates, minimal activity is observed. The pre-incubation with molecular sieve powder thus seems to have dried the enzyme to such an extent that it had a negative effect on its activity. The use of molecular sieves over longer periods of time should therefore be carefully considered as they may dehydrate and thereby inactivate the enzyme in time.

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