Highly concentrated water-in-oil emulsions as novel reaction media for protease-catalysed kinetically controlled peptide synthesis

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High-internal-phase-ratio-emulsions (HIPREs) or gel emulsions, formulated with a large amount of water (80.0–99.5% w/w), were investigated as reaction media for α-chymotrypsin-catalysed peptide synthesis under kinetic control using Ac-L-Phe-OEt and H-L-Leu-NH_2 as model substrates. Both the initial reaction rate and dipeptide yield were examined as a function of the structure of the non-ionic polyoxyethylene alkyl ether type surfactant, alkyl chain length of the oil component, temperature and aqueous buffer content. Dipeptide yields of 70% were achieved in gel emulsions formulated with 90% w/w aqueous buffer. In these systems, the reaction performance was found to be independent of the gel emulsion system (*i.e.* surfactant and oil) and therefore of the water–oil interfacial tension. Interestingly, α-chymotrypsin showed superactivity at surfactant concentrations ranging between 0.2 and 0.8% w/w, that is, at 99.5 and 98.0% w/w water content, respectively. Furthermore, high dipeptide yields (90–94%) were achieved in the gel emulsions studied at very high substrate concentrations and thus with undissolved reactants. Under these conditions, examples of α-chymotrypsin-catalysed dipeptide synthesis on an analytical and preparative scale were conducted.

Introduction

The use of enzymes in organic media has been widely investigated and numerous successful syntheses have been described.[1] Organic solvents enhance the solubility of hydrophobic substrates and enzymes exhibit striking new properties such as improved stability and selectivity.[2] However, recent trends in chemical industrial processes, legislation and public awareness of environmental concerns severely restrict the use of many traditional organic solvents.[3] Hence, solvent-free reactions[4] or reactions in solution using water[5] or supercritical CO_2[6] are the systems of choice. Among them, solvent-free and water are preferred as reaction media. The reason is that their use contributes to increasing the sustainability of the processes as they are safe, environmentally friendly and inexpensive compared to organic media.

One of the major drawbacks of water as a reaction medium is the poor solubility of hydrophobic compounds. This problem has usually been circumvented by adding water-miscible organic solvents to the aqueous reaction mixture.[7] Aqueous–organic cosolvent mixtures have been extensively used in enzyme-catalysed processes.[8] Nevertheless, 20–30% v/v cosolvent concentration is usually not enough for substrate solubility and often detrimental to the enzyme activity and stability. Alternatively, high water content colloidal systems such as vesicles, oil-in-water emulsions and high-internal-phase-ratio-emulsions (HIPREs), although used occasionally in organic synthesis,[9] have been rarely studied in biocatalysis.[10] In particular, the application of HIPREs or water-in-oil (W/O) gel emulsions in enzymatic reactions remains heretofore unexplored.

W/O gel emulsions are liquid–liquid dispersions with an internal phase volume fraction (water) larger than 0.74, the critical value of the most compact arrangement of spheres of equal radius.[11] Hence, the structure of gel emulsions consists of close-packed water droplets with radii typically of a few microns, separated by a thin film of continuous phase, a type of structure that resembles gas–liquid foams. Their rheological properties range from elastic (solid-like) to viscoelastic, depending on composition variables and temperature. In non-ionic polyoxyethylene alkyl ether type surfactants they form at temperatures above the hydrophile–lipophile balance (HLB) temperature or phase inversion temperature (PIT) of the corresponding system.[12] The HLB temperature is the temperature at which the non-ionic surfactants change the preferential solubility from water to oil.[13]

The advantages of gel emulsions as reaction media are: a) they can dissolve and/or solubilize large quantities of hydrophilic and hydrophobic compounds, b) they can be formulated with a large amount of water (*i.e.* as much as 99% w/w) and very low surfactant and oil concentrations (<5%), making them very attractive from the environmental point of view and facilitating the subsequent reaction work up and product purification, c) they have extremely large interfacial areas. Furthermore, they have the ability to maintain solid particles in suspension,[14] preventing their sedimentation, improving mixing and efficiently creating the necessary liquid phase in reactions with predominantly solid substrate particles (*i.e.* solid-to-solid systems).[15] This also may avoid continuous agitation of the medium during the reaction.[16]

In recent years gel emulsions have received a great deal of attention for polymerisation reactions to obtain solid foams, composites, latexes, *etc.*,[17] and in the chemical synthesis of surfactants derived from arginine.[16] Herein, we have endeavoured to apply gel emulsions as reaction media for protease-catalysed kinetically controlled peptide bond formation. The kinetic formation of amide bonds is based on the ability of serine- or cysteine-proteases to catalyse the acyl-transfer reaction between the C^{α} ester of an amino acid or peptide **1** and the N^{α} amino group of another amino acid or peptide derivative **2** (Scheme 1).[18] Proteases are not ideal transferases and the acyl-enzyme intermediate formed **3** can be deacylated by water as well. Usually, the rate of hydrolysis v_h is much lower than that of the

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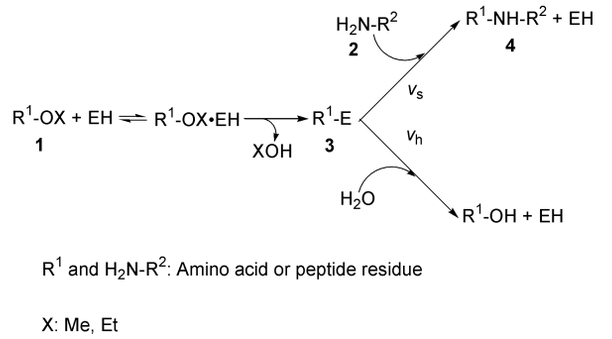

Scheme 1 Kinetically controlled enzymatic peptide synthesis. EH: serine or thiol protease, $v°_h$: initial rate of hydrolysis and $v°_s$: initial rate of synthesis.

aminolysis v_s and, consequently, the product **4** accumulates in the reaction media far above the thermodynamic equilibrium concentration. Then, the reactions can be performed in predominantly aqueous systems such as aqueous–organic cosolvent mixtures. The organic solvent is added merely to improve the substrate solubility. Hence, gel emulsions can be regarded as alternative reaction media to aqueous–organic cosolvent systems. To demonstrate this, in the present paper we studied the α-chymotrypsin-catalysed acyl-transfer reaction between Ac-L-Phe-OEt and H-L-Leu-NH_2, as a model, in gel emulsion media. Gel emulsions were tested in both dilute and high substrate concentrations. Finally, examples of dipeptide syntheses on an analytical and gram scale were performed under the best synthetic conditions.

Results and discussion

The gel emulsions prepared in this work consisted of an oil component of heptane, octane, dodecane, tetradecane or hexadecane, a surfactant of polyethoxylated non-ionic type, $C_{12}E_4$, $C_{14}E_4$, $C_{16}E_4$ or $C_{18}E_2$ (C_mE_n, where m is the alkyl chain length of the surfactant and n is the degree of ethoxylation) as dispersing agent and Tris-HCl 50 mM pH 7.8 aqueous buffer solution as internal phase. For experimental convenience the kinetically controlled peptide bond formation between Ac-L-Phe-OEt and H-L-Leu-NH_2 catalysed by α-chymotrypsin (CT) was selected as a model reaction. First, dilute solutions of 20 mM Ac-L-Phe-OEt and 30 mM of H-L-Leu-NH_2 were used for the sake of comparison with similar syntheses performed in our lab.[19,20]

Prior to any enzymatic reactions the stability of gel emulsions in the presence of substrates at 25 °C was determined for the different aqueous buffer solution–non-ionic surfactant–oil systems. To this end, gel emulsions formulated with both 90 and 98% w/w aqueous buffer were studied. In all cases the gel emulsions were stable (*i.e.* without macroscopic phase separation) for a period longer than 24 h, which was considered adequate for the reaction time. Furthermore, the poorly water soluble acyl-donor component, Ac-Phe-OEt, was dissolved in the oil phase of the gel emulsion system.

Effect of the oil and surfactant structures

The dipeptide yield and initial reaction rate ($v°$) for the model reaction were examined as a function of the oil chain length and structure of the polyethoxylated surfactant in gel emulsions consisting of aqueous buffer solution 90% w/w, surfactant 4% w/w and oil 6% w/w (*i.e.* aqueous buffer solution : surfactant : oil 90 : 4 : 6). The results obtained are summarized in Table 1. Overall, inspection of the data indicates that both the initial reaction rate and the product yield were independent of the nature of both oil and surfactant. The most striking result was the product yields (70%) obtained in these systems with 90% w/w of aqueous buffer, comparable to the 77 and 71% obtained in 50 : 50 v/v and 30 : 70 v/v dimethylformamide–buffer mixtures at 25 °C, respectively.[20] Interestingly, it was

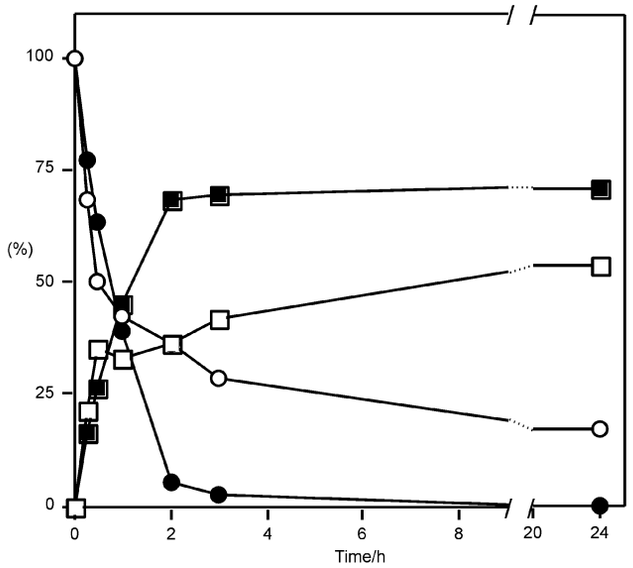

Fig. 1 Synthesis of Ac-L-Phe-L-Leu-NH_2 catalysed by α-chymotrypsin. Reaction time course of Ac-L-Phe-OEt (●, ○) and Ac-L-Phe-L-Leu-NH_2 (■, □) in Tris-HCl 50 mM pH 7.8 buffer–$C_{14}E_4$–octane 90 : 4 : 6 gel emulsion system (filled symbols) and pure Tris-HCl pH 7.8 50 mM buffer (empty symbols). Conditions in gel emulsions are described in the Experimental section. In pure aqueous buffer solution: Ac-L-Phe-OEt (0.1 mmol, 0.024 g) and H-L-Leu-NH_2 (0.15 mmol, 0.020 g) were suspended in Tris-HCl 50 mM pH 7.8 buffer (5 g). α-Chymotrypsin (20 μl of a 334 μg cm^{-3} solution, giving 1.3×10^{-3} mg g^{-1} in the final reaction mixture) was added and the reaction was placed in a reciprocal shaker at 25 °C.

evidenced by optical microscopy that the product precipitated from the reaction mixture and a homogeneous suspension of solid particles was formed co-existing with droplets of gel emulsion.

Table 1 also shows the calculated hydrophile–lipophile balance (HLB) temperatures for each system. It is well known that the closer the temperature to the HLB temperature of the aqueous buffer–surfactant–oil system the lower the water–oil interfacial tension.[21] Then, at 25 °C it is expected that the aqueous buffer–$C_{14}E_4$–hexadecane and aqueous buffer–$C_{18}E_2$–octane 90 : 4 : 6 systems exhibit the lowest and the highest water–oil interfacial tension, respectively. Our findings suggest that in the gel emulsion systems tested the reaction performance was not affected by this interfacial parameter.

For comparison's sake, Fig. 1 depicts the reaction progress curves of Ac-L-Phe-OEt consumption and dipeptide production for the model reaction in aqueous buffer solution and in an aqueous buffer–$C_{14}E_4$–octane gel emulsion system. One can see that whereas the initial reaction rate was rather similar in both reaction media, the product yield was higher in gel emulsion (70%) than in aqueous buffer solution (54%). In the latter case, the time course data show that the reaction progress slowed down steadily to 83% substrate conversion. A visual inspection of the reaction mixture in aqueous buffer solution revealed that Ac-L-Phe-OEt was not dissolved completely so that, after sonication, part of it remained as fine solid particles dispersed in the medium. Furthermore, a precipitate of product appeared during the reaction. On the basis of literature data,[22] it seems likely that the enzyme molecules may be irreversibly inactivated by its adsorption onto the solid–liquid interface.[23] In the gel emulsion systems the product formed also precipitated, even though no enzyme deactivation was observed during the reaction. This suggests that these systems may prevent the adsorption of the enzyme onto the solid particles, avoiding its inactivation.

Influence of temperature

In gel emulsions temperature can affect the reaction performance in three ways: a) by changing the catalytic rate of the

Table 1 Synthesis of Ac-L-Phe-L-Leu-NH_2 catalysed by α-chymotrypsin in Tris-HCl 50 mM pH 7.8 buffer–surfactant–oil 90 : 4 : 6 gel emulsion systems. Influence of oil chain length and surfactant structure on the initial reaction rate and dipeptide yield at 25 °C

Oil	Surfactant	Initial reaction rate/ μmol min^{-1}	Dipeptide yield (%)	HLB temperature[a]/ °C
Heptane	$C_{14}E_4$	0.7	67	2.0
Octane	″	1.2	71	4
Dodecane	″	1.3	70	14.0
Tetradecane	″	1.3	70	19.0
Hexadecane	″	1.3	70	22.0
Octane	$C_{12}E_4$	1.2	68	16.6
″	$C_{16}E_4$	1.2	68	−5.6
″	$C_{18}E_2$	1.2	68	−65

[a] Calculated following the equation reported by H. Kunieda and K. Shinoda, *J. Colloid Interface Sci.*, 1985, **107**, 107.

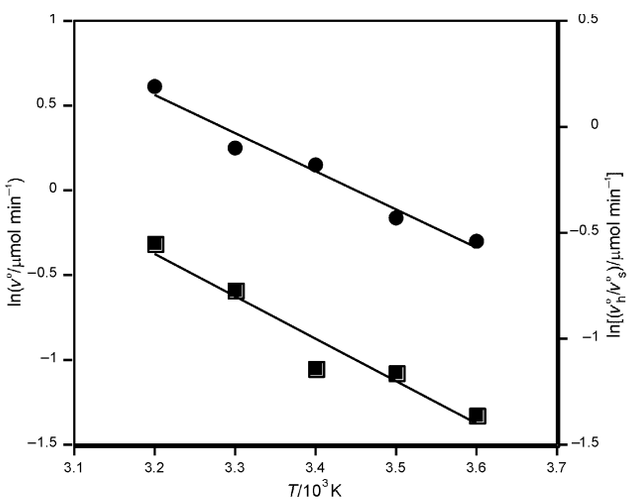

Fig. 2 Arrhenius plot of the $v°$ (●) and $v°_h/v°_s$ (■) for the α-chymotrypsin-catalysed synthesis of Ac-L-Phe-L-Leu-NH_2 in Tris-HCl 50 mM pH 7.8 buffer–$C_{14}E_4$–octane 90 : 4 : 6 gel emulsion system. Conditions were Ac-L-Phe-OEt (0.1 mmol, 0.024 g), H-L-Leu-NH_2 (0.15 mmol, 0.02 g), octane (0.3 g), $C_{14}E_4$ (0.2 g), Tris-HCl 50 mM pH 7.8 buffer (4.52 g) and α-chymotrypsin (1.3×10^{-3} mg g^{-1} mixture).

enzymatic reaction, b) by varying the rate of reactant diffusion and c) by altering the water–oil interfacial tension and consequently the stability of the system. As demonstrated in the aforedescribed experiments, the model reaction in gel emulsions was independent of the interfacial tension. Thus, since the reaction medium was not stirred during the synthesis, it may be thought that changes in the reaction temperature must influence the rate of enzymatic reaction and/or substrate diffusion.

The effect of temperature on initial reaction rate and dipeptide yield was examined in a range between 5 and 35 °C in aqueous buffer–$C_{14}E_4$–octane 90 : 4 : 6 gel emulsion. The choice of this system was made considering that the reaction temperature must always be above the HLB temperature of the system (4 °C) to ensure the formation of the water-in-oil gel emulsion. In addition, prior to any enzymatic reaction the formation of the gel emulsions and their kinetic stability were ascertained for each reaction temperature. Fig. 2 depicts both $v°$ and the ratio between the initial rates of hydrolysis and synthesis ($v°_h/v°_s$) plotted against the temperature in an Arrhenius type plot. The activation energy calculated was 19 kJ mol^{-1}, in good agreement with the same enzymatic reaction performed in aqueous buffer–cosolvent mixtures (22 and 13 kJ mol^{-1} for 40 and 30% v/v dimethylformamide, calculated from the data of ref. 20).[20,24] Moreover, $\ln(v°_h/v°_s)$ decreased linearly as the reaction temperature was raised. This indicates that the higher the reaction temperature the lower the dipeptide yield, which agreed with the observed values: 77 and 59% dipeptide yield at 5 and 35 °C, respectively. The same effect of temperature on the dipeptide yield was observed in organic media at low water content,[25] in water–cosolvent mixtures[20] and in frozen aqueous solutions at temperatures as low as −35 °C.[26] These features may support the conclusion that the reaction rate was controlled by the enzymatic catalysis and that the diffusion was fast and not rate-limiting. Furthermore, the results may also indicate that there was no change in the rate-determining step of the enzymatic catalysis or in the enzyme conformation[27] either in the gel emulsion system or induced by changes in the water–oil interfacial tension.

Influence of aqueous buffer content

In the high water-rich region of a gel emulsion system and at a constant oil : surfactant ratio, the water–oil interfacial area decreases as the water content is raised.[28] Assuming that the reaction takes place mainly in the interface areas, we hypothesise that the smaller the interfacial area, the lower the enzymatic reaction rate.

To test that, we examined the effect of aqueous buffer content on the reaction rate and dipeptide yield in a range between 80.0 to 99.5% w/w using an aqueous buffer–$C_{14}E_4$–octane gel emulsion system. It is noteworthy that at 99.5% w/w the gel emulsion was not formed and a dilute oil-in-water emulsion appeared instead. Furthermore, owing to the fact that the nucleophile was mainly located in the aqueous phase its concentration varied with the water content from 37.5 to 30.2 mM, respectively. Although this variation was considered not to have a significant effect on the enzymatic activity, it may affect the relative rates of hydrolysis ($v°_h$) and synthesis ($v°_s$) and therefore the dipeptide yield. In Fig. 3a a plot of the initial reaction rate against the aqueous buffer content is presented. One can see that the enzymatic activity increased sharply with the water content in the range from 95.0 to 99.5% w/w and then dropped at 100% w/w water. In the light of these results, there is strong evidence that CT showed superactivity. This phenomenon was observed in aqueous solutions of cationic surfactants[29] and it is well documented in reverse micellar systems.[30] However, to the best of our knowledge, no data are reported for polyoxyethylene non-ionic surfactants and gel emulsion systems such as the ones used in this work. In aqueous solutions, superactivity depends on the concentration and type of both surfactant and buffer.[29] Obviously, in our case, the higher the water content the lower the surfactant concentration so that the same shape as the curve in Fig. 3a was obtained by plotting the data against the $C_{14}E_4$ concentration. Hence, the enzymatic activity was influenced by the surfactant concentration rather than by the water content. Among the effects that induce enzymatic superactivity and reported by Spreti *et al.*,[29] in the present case, both hydrophobic and hydrogen bonding enzyme–surfactant interactions may play a remarkable role in view of the non-ionic nature of the surfactant used.

The behaviour of CT in gel emulsions was analysed considering the theoretical model developed by Viparelli *et al.*[31] for aqueous solutions of surfactants. Two assumptions were made in this case: first, the oil added is bound with the surfactant and second, the substrate acyl-donor (Ac-L-Phe-OEt) was mainly associated with the surfactant–oil phase. Hence, the experimental curve of initial reaction rate with the concentration of surfactant may be due to two physical situations: a) a higher efficiency of the enzyme in the bound water pseudo-phase (*i.e.* water around the polar heads of the surfactant) than in the free water and b) partition of the substrate between the dispersed phase (*i.e.* water) and the continuous oil phase. However, according to Spreti *et al.*[29] the enzyme superactivity cannot be related to substrate partitioning but to specific interactions with the surfactant and the microinterface areas. On the other hand, from the results obtained, nothing can be concluded concerning

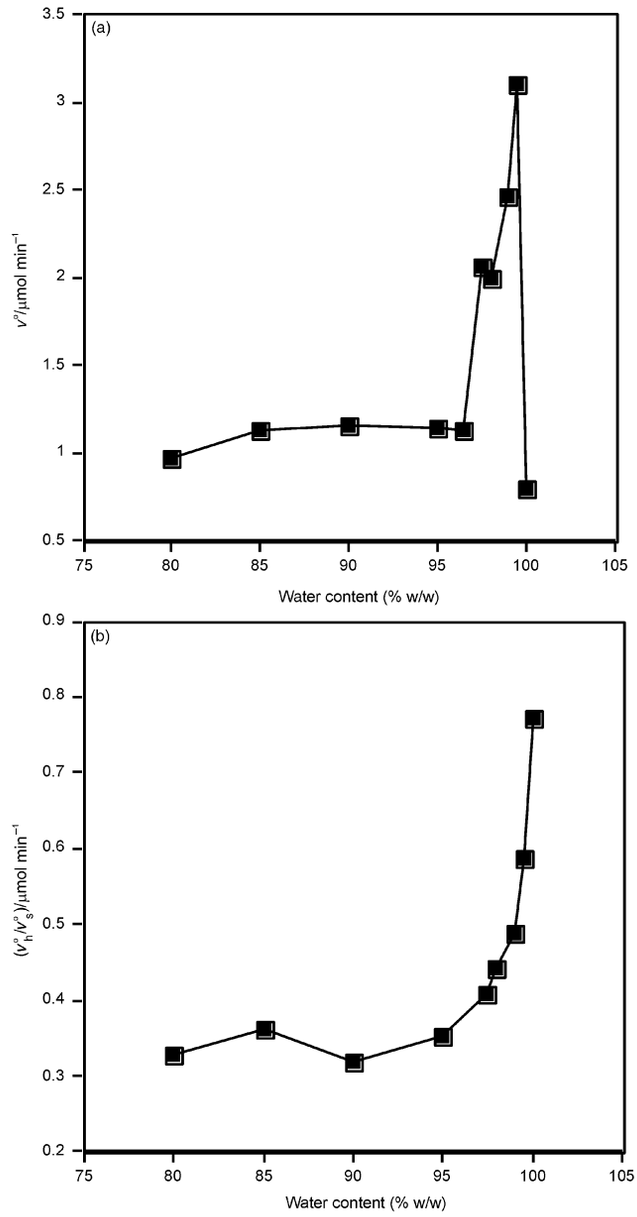

Fig. 3 Influence of the aqueous buffer concentration on v° (**3a**) and v°_h/v°_s (**3b**) for the α-chymotrypsin-catalysed synthesis of Ac-L-Phe-L-Leu-NH_2 in Tris-HCl 50 mM pH 7.8 buffer–$C_{14}E_4$–octane gel emulsion system. Conditions were Ac-L-Phe-OEt (0.1 mmol, 0.024 g), H-L-Leu-NH_2 (0.15 mmol, 0.02 g), octane (from 0.6 to 0.015 g), $C_{14}E_4$ (from 0.4 to 0.01 g), Tris-HCl 50 mM pH 7.8 buffer (from 4 to 4.975 g) and α-chymotrypsin (1.3×10^{-3} mg g^{-1} mixture).

the effect of the interfacial area on the reaction rate, but there is evidence that the superactivity phenomenon dominates the catalytic activity of the enzyme.

Concerning the product yield, Fig. 3b shows the plot of the ratio v°_h/v°_s against the water concentration. As can be seen, the higher the aqueous buffer content the faster was the hydrolysis rate of the acyl-donor ester substrate. As a consequence the dipeptide yields decreased from 70% at 80–95% w/w water to 63% at 99.5% w/w water. This fact may be related to the aforementioned decrease of the nucleophile concentration with the water content.

Synthesis in gel emulsion systems with suspended solid substrate particles

Reaction systems with partly undissolved substrates at very high concentrations (*i.e.* solid-to-solid systems) are emerging environmentally friendly alternatives to conventional reactions in solution. Many successful examples of dipeptide synthesis have been described which use either a kinetic or a thermodynamic approach.[4,32] In the kinetic approach, it has been reported that the addition of a surfactant was necessary to improve the reaction rate and product yield.[33] The surfactant has the ability to wet and solubilize the solid particles, facilitating the interaction with the enzyme and preventing its inactivation at the solid–liquid interface.

We wanted to investigate whether gel emulsions could also be useful systems in this case. Owing to their rheological properties, gel emulsions possess the ability to maintain solid particles in suspension, preventing their sedimentation. Furthermore, they may improve the mixing conditions while wetting effectively the hydrophobic substrate particles.

The main parameters affecting the reaction performance in "solid-to-solid" systems include: concentration of the liquid phase (*i.e.* gel emulsion in the present case), enzyme concentration and donor ester : nucleophile ratio.[15] Table 2 shows the effect of these parameters on the dipeptide yield for the model substrates. The reaction media were just stirred initially, when mixing the substrates with the gel emulsion, and after the addition of the enzyme during 30 s but not during the reaction. A visual inspection of the reaction mixtures, especially the ones of the first two entries of Table 2, under the optical microscope, revealed that the droplets of the gel emulsion coexisted with solid substrate particles in suspension. Interestingly, high dipeptide yields (90–92%) were achieved in 4 hours of reaction time, even when working with an equimolar concentration of donor ester and nucleophile. Excellent results were also obtained at gel emulsion concentrations as low as 20% w/w. In all cases the α-chymotrypsin concentration was in the range of other reported reactions in highly concentrated media.[33,34]

The scope of the proposed reaction medium was examined by studying other examples of dipeptide synthesis catalysed by chymotrypsin. Reactions were carried out in gel emulsions at very high substrate concentrations. The results obtained, presented in Table 3, showed the high yields achieved in all cases. Moreover, the enzymatic synthesis of Bz-L-Tyr-L-Phe-NH_2 was scaled up to grams, giving a 90% isolated yield with a purity of 99% by HPLC. Another important advantage of the gel emulsion systems is that they can be separated easily into two phases by the addition of plain water, by centrifugation or by changing the temperature. Moreover, the surfactant can be recovered into either the aqueous phase or the organic phase by changing the temperature to far below or above the HLB temperature. Thus, the work up of the reaction and the purification of the target product can be accomplished efficiently.

Table 2 α-Chymotrypsin-catalysed synthesis of Ac-L-Phe-L-Leu-NH_2 in Tris-HCl 50 mM pH 7.8 buffer–$C_{14}E_4$–octane 90 : 4 : 6 gel emulsions with suspended solid substrates at very high concentration. Effect of the amount of gel emulsion and enzyme concentration on the dipeptide yield. The substrate molar ratio was 1 : 1.5 acyl-donor : nucleophile unless otherwise stated

Amount of gel emulsion (w/w %)	Enzyme conc./g mol^{-1} donor ester	Reaction time/h	Dipeptide yield (%)
90	0.1	4	90
84	0.1	4	92
84[a]	0.1	2	89
70	0.1	2	91
53	0.1	48	56
20	0.1	24	14
53	2	4	94
20	2	4	92

[a] Equimolar substrate concentration.

Conclusions

The gel emulsion systems assayed in this work led to higher efficiencies for dipeptide synthesis catalysed by α-chymotrypsin

Table 3 Dipeptide synthesis catalysed by α-chymotrypsin in gel emulsions with suspended solid substrates at very high concentration. Conditions were: ester-donor (0.7 mmol), nucleophile (1.1 mmol), Tris-HCl 50 mM pH 7.8 buffer–$C_{14}E_4$–octane 90 : 4 : 6 to a final weight of 2.75 g and α-chymotrypsin (0.1 g mol^{-1} acyl-donor substrate)

Donor ester	Nucleophile	Reaction time/h	Conversion of donor ester (%)	Dipeptide yield (%)
Ac-Phe-OEt	Phe-NH_2	2	99	94
Bz-Tyr-OEt	Phe-NH_2	2; 24	90; 99	84; 92
Ac-Tyr-OEt	Leu-NH_2	5; 24	85; 99	79; 89
Bz-Tyr-OEt	Leu-NH_2	3; 24	96; 99	88; 94

at low and high substrate concentration. It was demonstrated that the enzymatic activity and yield were independent of the gel emulsion systems studied, differing in the HLB temperature and therefore in the water–oil interfacial tension. Furthermore, although the reaction media were not stirred continuously, the enzyme catalysis was rate-limiting rather than the reactant diffusion. There was strong evidence that CT showed superactivity at surfactant concentrations ranging between 0.2 and 0.8% w/w. The reaction system proposed here appears to be of general applicability, yet easy to prepare, in enzymatic transformations of water-insoluble substrates for both fundamental and applied uses. Most important, the W/O gel emulsions studied are as effective as 20–40% v/v dimethylformamide in water, while containing 90–95% w/w water. This will improve the safety of the process, minimizing the environmental impact.

Experimental

Materials

Polyoxyethylene nonionic surfactants with different chain lengths and an average ethylene oxide content of 2 and 4 mol per molecule ($C_{12}E_4$, $C_{14}E_4$, $C_{16}E_4$ or $C_{18}E_2$) were from Albright & Wilson (Barcelona, Spain). Heptane, octane, decane, dodecane and hexadecane analytical grade were from Sigma (St. Louis, MO, USA). α-Chymotrypsin (CT) (EC 3.4.21.1) from bovine pancreas (crystallized, lyophilised powder 350 U mg^{-1}, acetyl-L-tyrosine ethyl ester (ATEE) assay pH 8, 25 °C) was obtained from Merck (Darmstadt, Germany). *N*-Acetyl-L-phenylalanine ethyl ester (Ac-L-Phe-OEt), *N*-benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) and L-phenylalaninamide (H-L-Phe-NH_2) were from Sigma. *N*-Acetyl-L-tyrosine ethyl ester (Ac-L-Tyr-OEt) was from Nova Biochem (Läufelfingen, Switzerland) and L-leucineamide (H-L-Leu-NH_2) was from Bachem (Bubendorf, Switzerland). Acetonitrile HPLC isocratic grade was from Merck. All other reagents and buffers used were of analytical grade.

Methods

Preparation of gel emulsions. Gel emulsions were prepared as follows. The oil and the surfactant were mixed together. To this mixture, the amount of aqueous buffer solution was added dropwise while stirring, with a vortex mixer, continuously giving the final viscous white gel emulsion. All emulsions were prepared under the same experimental conditions. The rates of addition and stirring were kept constant to obtain reproducible emulsions.

Enzymatic reactions in gel emulsions. In a typical experiment, Ac-L-Phe-OEt (0.1 mmol, 0.024 g), octane (0.300 g) and the surfactant $C_{14}E_4$ (0.200 g) were mixed in a round bottomed, stoppered glass tube. The oil : surfactant weight ratio was kept constant at 3 : 2. In a different flask, H-L-Leu-NH_2 (0.15 mmol, 0.020 g) was dissolved in aqueous Tris-HCl 50 mM pH 7.8 buffer (4.500 g). This aqueous solution (the internal phase) was added dropwise to the oil and surfactant mixture (the external phase) while stirring at 25 °C. A highly viscous gel emulsion was formed (approx. 5 g of total weight). Immediately before adding the enzyme a sample was taken as time zero for the reactions. The synthesis was initiated by addition of CT (20 μl of a solution 334 μg cm^{-3}, giving 1.3×10^{-3} mg g^{-1} in the final reaction mixture) while mixing again for about 15 s. Then the reactions were placed in a thermostated water bath at 25 °C (unless otherwise stated) without any further agitation.

Sampling and HPLC analysis. Samples (50 mg) were withdrawn from the reaction medium, at different reaction times, and diluted with acetic acid (200 μl) to stop any further enzymatic reaction. Then, the mixture was dissolved with water–acetonitrile 50 : 50 containing trifluoroacetic acid (0.1% v/v) and analysed by HPLC. HPLC analyses were performed on a Merck-Hitachi Lichrograph system (Darmstadt, Germany) using a Lichrocart 250-4 HPLC cartridge, 250 × 4 mm filled with Lichrosphere® 100, RP-18, 5 μm (Merck). The solvent system was the following: solvent A: 0.1% v/v trifluoroacetic acid (TFA) in H_2O, solvent B: 0.085% v/v TFA in H_2O–CH_3CN 1 : 4; flow rate 1 ml min^{-1}, detection at 254 nm. The elution conditions for the separation of the components in each synthesis were the following: isocratic 40% v/v B (retention factors Ac-L-Phe-L-Leu-NH_2 1.3, Ac-L-Phe-L-Phe-NH_2 1.8, Ac-L-Phe-OEt 3.3, Ac-L-Phe-OH 0.8 and H-L-Phe-NH_2 0.3) gradient 20% B to 80% B v/v over 30 min (retention factors Bz-L-Tyr-L-Phe-NH_2 8.7, Bz-L-Tyr-OEt 9.9, Bz-L-Tyr-OH 5.9 and H-L-Phe-NH_2 1.0) gradient 10% B to 70% B over 30 min (retention factors Ac-L-Tyr-L-Leu-NH_2 5.5, Bz-L-Tyr-L-Leu-NH_2 9.8, Ac-L-Tyr-OEt, 7.7, Bz-L-Tyr-OEt 11.2, Ac-L-Tyr-OH 3.1, Bz-L-Tyr-OH 7.7 and H-L-Phe-NH_2 1.0). Peaks of products were identified by comparing the retention times with those of authentic samples or by high resolution electrospray mass spectrometry of the collected peaks from the HPLC. Quantitative analysis of products and reactants was performed from peak areas by the external standard method.

Initial reaction rates of substrate donor ester (Ac-L-Phe-OEt) consumption ($v°$/μmol min^{-1}), dipeptide product (Ac-L-Phe-L-Leu-NH_2) formation ($v°_s$/μmol min^{-1}), and hydrolysis product (Ac-L-Phe-OH) formation ($v°_h$/μmol min^{-1}) were calculated from the time progress curves.

Enzymatic reactions in gel emulsions at high substrate concentration. Two procedures were used to perform the reactions at high substrate concentration. For those reactions in which the amount of gel emulsion was higher than 50% w/w, the reactions were performed following the aforementioned procedure for low substrate concentration. When the amount of gel emulsion was lower than 50% w/w, it was more convenient to first mix the solid substrates and then add the gel emulsion previously prepared, stirring the final mixture thoroughly. However, this different mixing procedure gave the same results. Ac-L-Phe-OEt (1.2 mmol, 0.280 g) and H-L-Leu-NH_2 (1.8 mmol, 0.234 g) were used in an aqueous buffer Tris-HCl 50 mM pH 7.8–$C_{14}E_4$–octane 90 : 4 : 6 gel emulsion system. Reactions were initiated by adding 40 μl of an aqueous Tris-HCl 50 mM pH 7.8 buffer solution of CT (3.34 mg cm^{-3} to give 0.1 g CT mol^{-1} Ac-L-Phe-OEt, and 66.8 mg cm^{-3} to give 2 g CT mol^{-1} Ac-L-Phe-OEt). Then the reactions were placed in a thermostated water-bath at 25 °C without any further agitation.

Enzymatic synthesis of Bz-L-Tyr-L-Phe-NH_2 in gel emulsions. Bz-L-Tyr-OEt (3.7 mmol, 1.160 g) and H-L-Phe-NH_2 (5.6 mmol, 0.920 g) were suspended in 15 g of gel emulsion (aqueous buffer Tris-HCl 50 mM pH 7.8–$C_{14}E_4$–octane 90 : 4 : 6). Then, CT (160 μl of a solution of 4 mg CT cm^{-3}) was added (0.2 g of CT mol^{-1} acyl-donor substrate, 4.3×10^{-2} mg CT g^{-1} mixture). The product was isolated by pouring the gel emulsion into water and collecting the precipitate by filtration. The precipitate was washed successively with citric acid 5% w/v (2 × 50

ml), sodium bicarbonate 10% w/v (2 × 50 ml) and plain water (3 × 50 ml). The residue was freeze-dried to give 1.5 g of pure product (90% yield, 99.9% purity by HPLC). NMR data δ_H(300 MHz; d_6-DMSO) 9.06 (1H, s, OH), 8.40 (1H, d, *J* 7.5, NH), 7.93 (1H, d, *J* 7.5, NH), 7.76 (2H, d, *J* 7.5, NH_2), 7.51–7.04 (12H, m, Ph), 6.60 (2H, d, *J* 7.5, Ph), 4.57–4.48 (2H, m, 2(CH)), 3.01–2.82 (4H, m, 2(CH_2)). δ_C(75 MHz, d_6-DMSO) 172.6 (*C*O-NH), 171.0 (*C*O-NH_2), 166.3 (*C*-OH), 155.6 (Ph-*C*O-NH), 137.7–114.8 (11 signals, C aromatic), 55.2 and 53.6 (two signals, NH-*C*H-CO), 37.6 and 35.9 (two signals,*C*H_2). Electrospray-mass spectrum *m*/*z* $[M+H]^+$ 432.5; $C_{25}H_{25}N_3O_4$ requires 431.5.

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