# Peptide Synthesis Mediated by Immobilized and Viable Baker's Yeast in Reverse Micelles: Synthesis of Leucine Enkephalin Analogues

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Cells of baker's yeast (*Saccharomyces cerevisiae* NCIM 3305) immobilized in calcium alginate beads are found to be viable in reverse micelles of bis(2-ethylhexyl)sulphosuccinate sodium salt **1** in iso-octane for days and were used for the first time for peptide synthesis (in aerosol OT reverse micelles) using hydrophobic substrates.

Reverse micelles and microemulsion systems are being increasingly applied in enzyme-mediated organic syntheses, clinical analyses, drug-delivery systems and biotechnology.[1] Considering the importance of microbial cells for biotransformations,[2] experience with enzymology in reverse micelles has led to the evolution of 'microbiology in reverse micelles' as a new field for investigation. Both fundamental[3] and applied aspects[4] of microbial cells in these media are being actively studied. Previously we reported the use of baker's yeast in reverse micelles for the resolution of racemic α-amino acid esters.[4a] The stereoselectivity and substrate specificity for these reactions are very close to that of α-chymotrypsin, an enzyme widely used in peptide synthesis.[5] It occurred to us that baker's yeast could also be employed for peptide synthesis. However, baker's yeast possesses several proteases and also a carboxypeptidase. The amidase activity of these enzymes could cause a secondary hydrolysis of the growing peptide. In the case of α-chymotrypsin this problem has been solved either by chemical modification of the enzyme[6] or by using a high concentration (40–50%) of a dipolar aprotic organic solvent like *N,N*-dimethylformamide in the reaction medium.[7] These approaches cannot be used in the case of microbial cells as the products of cell lysis would excessively contaminate the desired peptide. Here we report a simple way to achieve peptide bond formation without significant side reactions using whole cells of baker's yeast (*Saccharomyces cerevisiae* NCIM 3305) immobilized in calcium alginate beads in a reverse micellar reaction medium.† To the best of our knowledge, this is the first report of peptide synthesis mediated by whole cells of baker's yeast in such a medium.

† Yeast cells were grown as described in ref. 4*a* and were immobilized (5 g, wet, per 15 ml of 2% sodium alginate) as in ref. 9. Reactions were carried out with alginate beads (40 g, wet) suspended in reaction medium (100 ml). General protocol for peptide synthesis in reverse micelles as described in ref. 5*e* was followed. All amino acid derivatives used were of the L-configuration. The reactions were usually complete within 6–8 h. After filtration of the beads the reaction mixture was treated with Amberlyst ion-exchange resins. The neutral peptides were then obtained in 70–75% isolated yields after column chromatography over silica gel.

$$\underset{\mathbf{1}}{\text{Ac-Phe-OMe}} + \underset{\mathbf{2}}{\text{H-Leu-NH}_2} \rightarrow \underset{\mathbf{3}}{\text{Ac-Phe-Leu-NH}_2} \quad \text{(a)}$$

$$\underset{\mathbf{4}}{\text{Z-Ala-Phe-OMe}} + \text{H-Leu-NH}_2 \rightarrow \underset{\mathbf{5}}{\text{Z-Ala-Phe-Leu-NH}_2} \quad \text{(b)}$$

$$\underset{\mathbf{6}}{\text{Z-Gly-Gly-Phe-OMe}} + \text{H-Leu-NH}_2 \rightarrow \underset{\mathbf{7}}{\text{Z-Gly-Gly-Phe-Leu-NH}_2} \quad \text{(c)}$$

$$\underset{\mathbf{8}}{\text{Z-Tyr-OMe}} + \underset{\mathbf{9}}{\text{H-Gly-Gly-Phe-Leu-NH}_2} \rightarrow \underset{\mathbf{10}}{\text{Z-Tyr-Gly-Gly-Phe-Leu-NH}_2} \quad \text{(d)}$$

**Scheme 1**

When suspended in a reverse micellar medium consisting of bis(2-ethylhexyl) sulphosuccinate sodium salt (Aerosol/OT) in iso-octane (0.1 M) and small amounts of aqueous buffer, the immobilized cells were found to be viable for weeks. Such immobilized and viable cells of baker's yeast were used for peptide synthesis in Aerosol/OT reverse micelles (reactions a and b, Scheme 1). This methodology was successfully employed in the synthesis of biologically important enkephalin analogues **7** and **10**[8] (reactions c and d, Scheme 1).

Fig. 1 shows the effect of the water content ($W_0$) of the micellar medium‡ on the yield of peptide product (HPLC analysis) for the reactions a, b and c.§ As the amount of water in the medium increases the hydrolysis of the ester becomes important. Presumably the reactions proceed through an acyl enzyme intermediate as in the case of the serine proteases[5] and the competition between leucinamide and water for nucleophilic attack on the acyl enzyme, decides the product distribution between peptide formation and hydrolysis of the ester substrate.¶

Reaction a was also performed in aqueous medium (glycine buffer, pH 10) in which the peptide **3** was obtained in 40% yield. The increased yield of peptide product (80%) in reverse micelles at low water content is most probably due to a high local concentration of hydrophilic leucinamide in the alginate beads‖ and the low water content of the medium. For reactions b and c no peptide product was observed in the absence of surfactant, only a slow hydrolysis of the ester substrate was observed.** The reverse micelles probably form an interface between gel matrix and the outer hydrocarbon solvent and facilitate efficient transport of lipophilic reagents across this interface without destroying the cells to a great extent. We have recycled the alginate beads ten times over a period of two weeks without change in product distribution, although the reaction times increased owing to slow depletion of the enzymes.

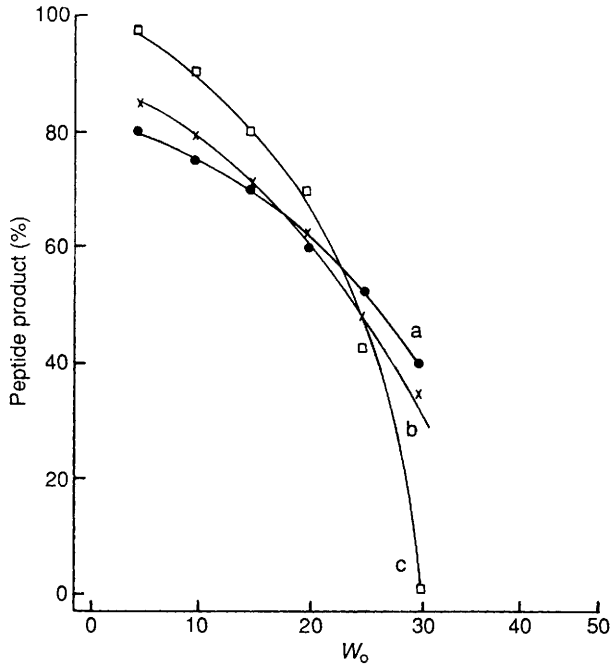


**Fig. 1** Dependence of water content on micellar medium of peptide bond formation for reactions a, b and c at room temperature. The product formation is expressed in terms of ester substrate. [Aerosol OT] = 0.1 M in iso-octane, $pH_{st}$ 10 (pH of stock buffer solution, glycine–NaOH, 50 mM), [Ester] = 1 mM overall, [H-Leu-$NH_2$·HCl] = 2 mM overall. $W_0$ = [$H_2O$]/[Aerosol OT]

‡ $W_0$ is the molar ratio between water and surfactant ($W_0$ = [$H_2O$]/[Aerosol OT]).

§ All compounds gave satisfactory spectroscopic and analytical data. 1H NMR ([$^2H_6$]DMSO, 300 MHz, $\delta_H$, ppm) **3**: 0.82 (dd, 6H, Leu-Me, *J* 6.8 Hz), 1.47 (m, 3H, Leu-CH and $CH_2$), 1.99 (s, 3H, COMe), 3.15 (d, 2H, Phe-β-$CH_2$, *J* 6.0 Hz), 4.23 (m, 1H, α-CH), 4.89 (m, 1H, α-CH), 5.92 (s, 1H, acetyl NH), 7.20 (s, 2H, Leu-$NH_2$), 7.25–7.33 (m, 5H, Ar), 8.02 (m, 1H, peptide bond NH); **5**; 0.84 (dd, 6H, Leu-Me, *J* 6.8 Hz), 1.11 (d, 3H, Ala-Me, *J* 6.8 Hz), 1.46 (m, 3H, Leu-CH and $CH_2$), 3.05 (d, 2H, Phe-β-$CH_2$, *J* 6.0 Hz), 4.01 (m, 1H, α-CH), 4.21 (m, 1H, α-CH), 4.49 (m, 1H, α-CH), 5.05 (s, 2H, Ar-$CH_2$-O), 7.15 (s, 2H, Leu-$NH_2$). 7.23–7.35 (m, 10H, Ar-H), 7.48 (m, 1H, urethane NH), 8.06 (m, 2H, peptide bond NH); **7**: 0.85 (dd, 6H, Leu-Me, *J* 6.7 Hz), 1.45 (m, 3H, Leu-CH and $CH_2$), 2.95 (d, 2H, Phe-β-$CH_2$, *J* 6.0 Hz), 3.60 (s, 2H, Gly-CH), 3.66 (s, 2H, Gly-CH), 4.18 (m, 1H, α-CH), 4.52 (m, 1H, α-CH), 5.03 (s, 2H, Ar-$CH_2$O), 6.97 (s, 2H, Leu-$NH_2$), 7.20–7.35 (m, 10H, Ar-H), 7.47 (m, 1H, urethane NH), 8.17 (m, 3H, peptide bond NH); **10**: 0.84 (dd, 6H, Leu-Me, *J* 6.7 Hz), 1.45 (m, 3H, Leu-CH and $CH_2$), 2.90–3.02 (m, 4H, Tyr + Phe-β-$CH_2$), 3.60 (s, 2H, Gly-CH), 3.66 (s, 2H, Gly-CH), 4.20 (m, 1H, α-CH), 4.48–4.53 (m, 2H, α-CH), 5.07 (s, 2H, Ar-$CH_2$O), 5.27 (s, 1H, Ar-OH), 6.97 (s, 2H, Leu-$NH_2$), 6.48–7 42 (m, 14H, Ar-H), 7.48 (m, 1H, urethane NH), 8.20 (m, 4H, peptide bond NH).

¶ Apart from the hydrolysis of the ester no other byproduct was observed in the HPLC analysis.

‖ HPLC analysis for leucinamide indeed shows that addition of alginate beads causes a 30–40% decrease in its concentration in supernatent micellar media.

** Since the substrates were insoluble in iso-octane, mixtures of iso-octane–chloroform, carbon tetrachloride or ethylacetate were employed. Other reaction conditions were unchanged.

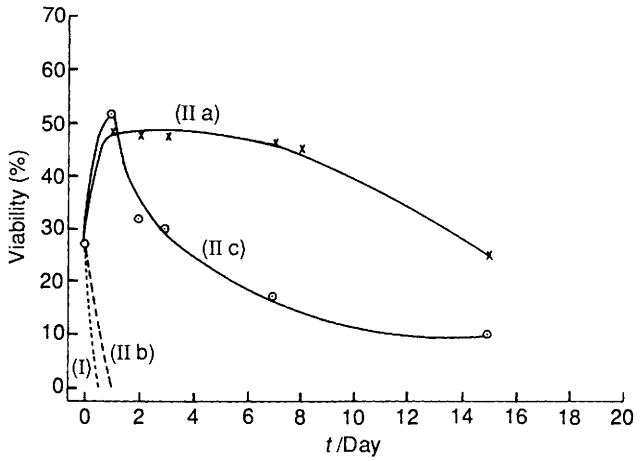


**Fig. 2** Viability of yeast cells (%) under different microenvironmental conditions (room temp., no added nutrients); (I) Free yeast cells in reverse micelles, [Aerosol OT] = 0.1 M in iso-octane, $W_o = 20$, $pH_{st}$ 7 (glycine buffer, 50 mM). (II) Immobilized yeast cells suspended in (*a*) aqueous buffer (glycine buffer, 50 mM, pH 7); (*b*) reverse micelles of CTAB, [CTAB] = 0.1 M in 1 : 1 (v/v) chloroform–iso-octane, $W_o = 20$, $pH_{st}$ 7; (*c*) reverse micelles of Aerosol OT, conditions as for (I)

Fig. 2 shows the viability of the immobilized yeast cells in reverse micellar suspension. While the free cells are completely destroyed in a few hours (I) the immobilized cells are able to survive in such a toxic medium for a long time even in the absence of any added nutrient. For the first 24 h the yeast cells behave in the reverse micellar medium as if they are in water and are able to divide. Cell viability increases from 28 to 50% and the cell count per bead increases from $5 \times 10^7$ to $7 \times 10^7$ in 24 h and $8 \times 10^7$ in 48 h. Although the viability of the cell starts decreasing faster than that in water this decrease is not too dramatic. The data in Fig. 2 cover a period of two weeks but we have observed that many of the cells were still visible after one month. The viability of the cells was lost very quickly when a positively charged surfactant, hexadecyltrimethylammonium bromide (CTAB) was used for preparation of the reverse micelles (IIb). It thus appears that the immobilized cells are protected in the present case due to electrostatic repulsion between negatively charged groups of the surfactant and the alginate beads. This phenomenon is general as has been observed in our preliminary studies with some bacteria and fungi.

Our methodology thus offers an alternative to aqueous or biphasic systems for microbial transformations. Here, the microbial cells are viable, conditions can be 'tailored' to increase the yield of desired product and biotransformations with lipophilic substrates can be performed easily. Baker's yeast provides perhaps the cheapest 'biological catalyst' for peptide synthesis. Work is in progress on the application of this technique to the synthesis of opioid peptides, dynorphins and neurokinins, and the use of viable cells in reverse micells for stereospecific reductions, oxidations *etc.*, using lipophilic substrates.

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