Non-thermal effects of microwaves on protease-catalyzed esterification and transesterification

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Abstract—The non-thermal effects of microwave irradiation on enzyme-catalyzed reactions have been evaluated by keeping the reaction temperature constant during irradiation. Subtilisin-catalyzed transesterification and a-chymotrypsin-catalyzed esterification have been carried out in six solvents of differing polarities and at three different temperatures. In all cases, microwave irradiation was found to increase the initial reaction rates by 2.1-4.7 times at all hydration levels. It is also shown that microwave irradiation can be used in conjunction with other strategies (like pH tuning and salt activation) for enhancing initial reaction rates.

1. Introduction

Using microwaves to promote reaction rates of chemical reactions has become fairly routine.1,2 Their application in enzyme-catalyzed reactions is relatively limited.3-7 In the latter case, reactions have been studied mostly in non-aqueous media. This is largely due to two factors. Firstly, most of the important synthetic applications are carried out in non-aqueous media.7,8 Secondly, unlike in aqueous buffers, enzymes display high thermal stability in such media.8,9 In general, it is believed that the reactions are accelerated since the molecules absorb energy by two modes; dipole rotation and ionic movement. As pointed out by Kabza et al.,10 ‘these proposed mechanisms do not explain some unusually high yields and quick conversions reported’ even in the case of chemical transformations. Most of the studies have been made using domestic ovens and thermal and non-thermal effects have not been distinguished. However, Parker et al.9 have used a microwave oven equipped with infra-red continuous feedback temperature control system and observed that ‘irradiating a hydrated lipase enzyme suspended in organic media using microwaves (2.45 GHz, 508C) enhanced the reaction rate by 2-3 fold over classical heating’. Porcelli et al.11 have used 10.4 GHz microwave radiation of elevated intensity using a waveguide system which allowed thermal control and accurate microwave dosimetry. The objective of this work was to study non-thermal effect of microwaves on enzyme inactivation, in a temperature range of 70-908C, in aqueous medium. The latter choice was possible since the enzymes that they used (S-adenosylhomocysteine hydrolase and 5'-methylthioadenosine phosphorylase) were from a thermophile Sulfolobus sulfataricus. It was found that the inactivation (due to microwaves) kinetics did not depend upon protein concentration. The significant inactivation of enzymes due to exposure to radiation was in agreement with conformational changes revealed by fluorescence emission spectra and far ultraviolet CD spectra. More recently, the same group has looked at inactivation of alcohol dehydrogenase from the same source and confirmed that a non-thermal effect was responsible for enzyme inactivation.12

In the present work, we have chosen two proteases, subtilisin and a-chymotrypsin, and investigated the effect of microwaves alone at fixed temperatures on the rates of esterification13 and transesterification14 reactions catalyzed by these enzymes.

2. Results and discussion

The proteases, subtilisin and a-chymotrypsin, are known to catalyze esterification and transesterification reactions in organic solvents, under low water conditions.7,8 It was found that in the case of non-irradiated samples, the variation in the initial rates with solvents of different polarities, obtained with both enzymes followed the same order as observed by Zaks and Klibanov14 in their seminal work with these two enzymes (Tables 1 and 2). In the case of microwave-irradiated samples as well, the same trend was observed. As observed by these workers, in the case of subtilisin-catalyzed reaction in t-amyl alcohol, the rate was much higher than even in n-octane. A control was run to confirm that t-amyl alcohol did not act as a substrate in the reaction. For a-chymotrypsin, the initial rates increased with increase in log P as observed by Reslow et al. at
Table 1. Subtilisin-catalyzed transesterification reaction

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial reaction rate (μmol h⁻¹)</th>
<th>Change in rate Vm/Vc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classical Vc</td>
<td>Microwave Vc</td>
</tr>
<tr>
<td>n-Octane (4.5)</td>
<td>2.20 3.50 6.41</td>
<td>6.50 10.33 20.19</td>
</tr>
<tr>
<td>Toluene (2.5)</td>
<td>0.15 0.26 0.55</td>
<td>0.63 1.08 2.46</td>
</tr>
<tr>
<td>t-Amyl alcohol (0.89)</td>
<td>2.13 3.56 6.50</td>
<td>7.63 13.22 30.55</td>
</tr>
<tr>
<td>Acetonitrile (20.33)</td>
<td>0.15 0.25 0.54</td>
<td>0.45 0.80 1.74</td>
</tr>
<tr>
<td>Dioxane (21.1)</td>
<td>0.01 0.02 0.04</td>
<td>0.01 0.04 0.10</td>
</tr>
</tbody>
</table>

Values in brackets represent the log P values of these solvents, which is defined as the logarithm of the partition coefficient of a solvent between octanol and water.

Table 2. a-Chymotrypsin-catalyzed esterification reaction

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial reaction rate (μmol h⁻¹)</th>
<th>Change in rate Vm/Vc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classical Vc</td>
<td>Microwave Vc</td>
</tr>
<tr>
<td>n-Octane (4.5)</td>
<td>1436 3336 7382</td>
<td>5313 12410 27465</td>
</tr>
<tr>
<td>Toluene (2.5)</td>
<td>120 251 541</td>
<td>420 884 1940</td>
</tr>
<tr>
<td>t-Amyl alcohol (0.89)</td>
<td>58 95 193</td>
<td>166 277 571</td>
</tr>
<tr>
<td>Acetonitrile (20.33)</td>
<td>10.3 19.6 70</td>
<td>33 75 255</td>
</tr>
<tr>
<td>Dioxane (21.1)</td>
<td>1.4 2.5 6.3</td>
<td>3.5 6 15.1</td>
</tr>
</tbody>
</table>

Values in brackets represent the log P values of these solvents, which is defined as the logarithm of the partition coefficient of a solvent between octanol and water.

pH-tuned enzyme was used in all these cases.

| Values in brackets represent the log P values of these solvents, which is defined as the logarithm of the partition coefficient of a solvent between octanol and water.28

The values given are at the optimal water levels.

258C.15 In fact, the same trend was observed at all temperatures in the case of both non-irradiated and irradiated samples. Thus, with this enzyme, the rate in t-amyl alcohol was lower than that observed in n-octane for both non-irradiated and irradiated samples. In all the cases, microwave irradiation resulted in an increase in initial reaction rates as compared to the rates observed in controls (with classical heating) at the same temperature. This was observed at all levels of hydration of the system in the range of 0.05-0.5% (v/v) added water (data not shown). The optimum amounts of added water (for obtaining maximum initial reaction rates) were the same for microwave-irradiated and non-irradiated samples. The optimum amount was in the range of 0.2-0.3% (v/v) added water and was identical for a particular solvent at all the three temperatures used (data not shown).

It was observed (Tables 1 and 2) that the increase in initial reaction rates due to microwave irradiation was in a similar range at all the three temperatures with a particular solvent. Again, the exception was the reaction catalyzed by subtilisin in t-amyl alcohol where the rate enhancement in the case of irradiated sample (over classical heating) increased with increasing temperature. More importantly, the extent of increase did not correlate with any solvent parameter. Toluene and t-amyl alcohol seemed to be the best solvents for exploiting microwave assistance in enhancing rates of subtilisin-catalyzed reactions. In the case of a-chymotrypsin, n-octane was the best solvent, even for the microwave-assisted approach. It is also interesting to note that the increase in reaction rates observed is of the same order as reported by others, such as Lin and Lin, who used domestic microwave ovens without any temperature control.

The experiments discussed so far were done with pH-tuned enzymes.16 This is because the protonation state of the enzymes in organic solvents is decided by the pH of the aqueous buffer from which the enzymes are lyophilized. As a result of this 'pH memory',17 it is general practice to lyophilize enzymes from aqueous buffers at a pH corresponding to the optimum pH of the enzyme in aqueous medium. It was considered worthwhile to examine whether microwave irradiation had any effect on the pH memory. It was seen (Fig. 1) that at 258C (in n-octane), subtilisin showed 3.8 times increase in reaction rate after pH tuning, while microwave irradiation of pH-tuned enzyme increased the reaction rate by 4.3 times (over untuned enzyme with irradiation). Microwave irradiation of untuned enzyme, on the other hand, increased the reaction rate by 2.2 times (over non-irradiated sample). As the microwave irradiation increased the reaction rate by 2.9 times (Table 1) (as compared to the non-irradiated pH tuned enzyme), the effect of microwave and pH tuning are not cumulative.

Recently, it has been reported that lyophilizing the enzyme solution in the presence of KCl enhanced the rates of the enzymatic reactions carried out using such lyophilized powders in organic solvents.18 Figure 1 shows the effect of microwaves in conjunction with the use of this 'salt
Figure 1. Effect of pH-tuning and salt activation on the rate of transesterification by subtilisin in n-octane at 258°C. For pH tuning, subtilisin (100 mg) was dissolved in 10 ml of 20 mM Tris-HCl, pH 7.8 and the solution was lyophilized. The lyophilized powder was then suspended in organic solvents and the reactions carried out. For salt activation, 10 mg of the pH-tuned enzyme was added to 10 mg K\textsubscript{2}HPO\textsubscript{4} and 0.98 g KCl and the solid was dissolved in 40 ml of distilled water and the pH adjusted to 7.8. This solution was then lyophilized and used further as described in Section 3.

Figure 2. Effect of pH-tuning and salt activation on the rate of transesterification by subtilisin in t-amyl alcohol at 258°C. The experimental details are described in the legend to Figure 1 and in Section 3.
activation' strategy in the case of subtilisin-catalyzed transesterification in n-octane. The 'salt activation' effect was found to be in a similar range for microwave-irradiated samples (2.1 times over the irradiated enzyme lyophilized in the absence of salt) as for the non-irradiated samples (2.7 times over the non-irradiated enzyme lyophilized in the absence of salt). It should be added that the range of 'salt activation' observed by us is in agreement with the observation of Triantafyllou et al. and unlike more dramatic effects reported by another group. The corresponding results with t-amyl alcohol are shown in Figure 2. While the general trend was similar, the 'salt activation' effect for non-irradiated sample was 2.2 times and merely 1.2 times for the irradiated sample. It is also worth noting that effect of microwave irradiation on the pH-tuned enzyme was different for n-octane and t-amyl alcohol.
alcohol. While irradiation of the pH-tuned enzyme in n-octane increased the rate by 4.3 times as compared with the rate obtained by irradiation of the untuned enzyme; in t-amyl alcohol, the corresponding increase in rate was 7.6 times.

Figure 3 shows the corresponding data for a-chymotrypsin catalyzed esterification in n-octane. Microwave irradiation again enhanced the initial rates of transesterification for untuned enzyme, pH-tuned enzyme and salt-activated enzyme. Again, the increases were observed over the entire range of added water. Figure 4 gives similar data but when the esterification is carried out in toluene. The results are similar in both solvents which differ considerably in log P values.

These results clearly establish that increase in reaction rates observed in the case of microwave-assisted reactions are not due to thermal effects alone. The data also show that by combining some strategies, considerable enhancement in reaction rates can be obtained in non-aqueous enzymology. For example, whereas untuned subtilisin showed a transesterification rate of 0.5 mmol h\(^{-1}\) at 25°C in n-octane [with 0.3 % (v/v) added water], the salt-activated and pH-tuned subtilisin gave a microwave-assisted reaction rate of 11.76 mmol h\(^{-1}\) (with the same level of water). This is about 20 times increase in the initial rate. It is also interesting to note that medium effects dominate over other factors. The esterification rate in toluene in case of microwave-assisted reaction with pH-tuned and salt-activated a-chymotrypsin (1.10 mmol h\(^{-1}\)) is still much lower than just pH-tuned a-chymotrypsin in n-octane (5.31 mmol h\(^{-1}\)).

We are investigating whether other enzymes behave in a similar way during microwave-assisted reactions.

3. Experimental

a-Chymotrypsin, subtilisin Carlsberg, N-acetyl-L-phenylalanine (N-OAc-Phe) and N-acetyl-L-phenylalanine ethyl ester (N-OAc-Phe-OEt) were obtained from Sigma Chemical Co., USA. The organic solvents used in this work were of low water content (0.0075 % water (v/v)) and were further dried by gentle shaking with 3 Å molecular sieves (CDH, Mumbai, India) overnight before use.

3.1. Enzyme assays

The activity of a-chymotrypsin in organic solvents was measured by following the esterification of N-OAc-Phe with ethanol.\(^{11}\) The amount of N-OAc-Phe-OEt synthesized was monitored by HPLC using a Beckman C18 column, eluted with water/acetonitrile/acetic acid 55:40:5, and detection of absorbance at 258 nm. The activity of subtilisin in organic solvents was measured by following the transesterification of N-OAc-Phe-OEt with propanol.\(^{13}\) The amount of N-OAc-Phe-OPr synthesized was monitored by HPLC using a Beckman C18 column, eluted with water/acetonitrile/acetic acid 57.5:37.5:5, and detection of absorbance at 254 nm.

3.2. Treatment of enzymes

3.2.1. Classical heating. a-Chymotrypsin (10 mg) was added to the dried solvent, followed by addition of water. This was followed by addition of 118 mL of ethanol containing 4 mg of N-OAc-Phe, making up the final volume of the reaction mixture to 19 mL with the organic solvent and heating the preparation in a screw-capped vial at the desired temperature for 30 min in an orbital shaker (at 200 rpm). Subtilisin Carlsberg (10 mg) was added to the dried solvent, followed by addition of water. This was followed by addition of 75 mL of n-propanol containing 12 mg of N-OAc-Phe-OEt, making up the total volume of the reaction mixture to 1 mL and heating the preparation in a screw-capped vial at the desired temperature for 30 min in an orbital shaker (at 200 rpm).

3.2.2. Microwave irradiation. Identical substrate and enzyme were taken as above and placed in a quartz vessel. The whole reaction mixture was placed in the microwave oven (Model RM2001, Plazmatronika, Wroclaw, Poland, operating frequency 2.5 GHz). The oven had an in-built magnetic stirrer which was used to stir the reaction mixture. The temperature of the reaction mixture was set as desired and measured using a non-contact infra-red continuous feed-back temperature system.

All experiments were carried out in duplicate and values represent the average of two independent experiments. The difference in the individual readings in each set was less than 5%.

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References

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