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TECHNICAL NOTE

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A microwave-assisted microassay for lipases

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Abstract A quick and efficient two-step assay for monitoring and screening lipase activity that uses a microtitre plate is described.

Keywords Lipase assay · Microwave · Screening of lipases

Introduction

Lipases are versatile biocatalysts and are being increasingly widely employed for synthesis and enantiomeric resolution of large numbers of biochemically relevant molecules [1–3]. This has led to considerable interest in developing methods for detecting and measuring lipase activity in a wide variety of situations [4]. The most well-known assay uses a pH-stat method for measuring the hydrolysis of tributyrin [1]. The hydrolysis of *p*-nitrophenyl palmitate (*p*-NP) has been recently used to assay lipase activity in both aqueous and organic media [5, 6]. This colorimetric method monitors the release of *p*-nitrophenolate ions and is an attractive alternative to the hydrolysis of tributyrin as it is simple, fast and convenient. However, there are some experimental difficulties associated with this method. Turbidity from the substrate or the product has been mentioned as one interfering factor in some cases [7]. While many workers employ the assay in two-step format, there is no suitable method for terminating the reaction. Thus, overestimating the enzyme activity to varying degrees leads to irreproducibility of results [5–7]. The assay described here has been developed to address these issues. It incorporates the use of Triton X-100 in order to avoid turbidity, as suggested in an earlier work [7]. However, it uses microwaves to terminate the reaction. Finally, the method is adapted for use in a microtitre plate. This allows one to work with small volumes of reagents in a typical ELISA environment.

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Materials and methods

The lipases were gifts from Amano Enzymes Inc., Nagoya, Japan. *p*-NP was purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade.

Test tube format of lipase assay

The method used has been described previously [5] except that the reaction was monitored up to 30 min (instead of just 5 min). The reaction mixture (in 25 ml borosilicate test tube) consisted of 1.8 ml of buffer (0.1 M sodium phosphate containing 0.15 M NaCl and 0.5% v/v Triton X-100, pH 7.0), 0.2 ml of enzyme solution in 0.1 M sodium phosphate buffer, pH 7.0, and 20 µl of 0.05 M substrate (*p*-NP) in acetonitrile. The mixture was incubated for 30 min at 37 °C and after this the solutions were kept in a domestic microwave oven (along with a beaker containing a volume of the water sufficient to make the total volume of the liquid in the chamber up to 100 ml). This was done to avoid overheating the samples. The absorbance was read at 410 nm after irradiation for 30 s at a frequency of 2.45 GHz. One enzyme unit is defined as the amount of the enzyme that liberates 1 µmole of *p*-nitrophenol per min at pH 7.0 and 37 °C.

Microtitre format of lipase assay

The reaction mixture (0.22 ml in each well of microtitre plate) consisted of 0.1 ml of buffer (0.1 M sodium

phosphate containing 0.27 M NaCl and 0.9% v/v Triton X-100, pH 7.0), 0.1 ml of enzyme solution in 0.1 M sodium phosphate buffer, pH 7.0, and 20 µl of 0.005 M *p*-NP in acetonitrile. The mixture was incubated for 30 min at 37 °C, and then the solutions were kept in a domestic microwave oven and irradiated for 30 s as described above, and read at 410 nm.

Results and discussion

In general, two-step assays are considered more user-friendly than continuous measurement protocols. The format requires a termination step so that the readings can be measured afterwards within a reasonable time-span. Kilcawley et al [5] did not specify whether a continuous or two-step protocol was used while describing a colorimetric assay based upon *p*-NP hydrolysis. Other workers [6] have raised the issue of the termination step and rightly pointed out that even raising the pH to 11.0 by the addition of alkali is not always satisfactory. Recently, cooling the reaction system on ice has been suggested for terminating the enzymatic reaction [7]. Figure 1 shows that this fails to stop the reaction and the reaction continues as soon as the temperature is raised in order to measure the absorbance. Thus, the cooling step did not terminate the reaction. This was, of course, expected. However, the data in Fig. 1 shows that this approach could lead to a significant overestimation of enzyme activity.

The use of microwaves to stop unwanted enzymatic activities during food processing has been described [8, 9]. It has been mentioned that enzymes in aqueous media rapidly lose their activity when subjected to microwaves [9]. Figure 2 shows that exposing a lipase to microwaves for 30 s inactivates it completely. This inactivation could be used to terminate the assay reaction. Similar results were found with other lipases (results not shown). Figure 3 shows the standard curve for four different lipases in microtitre format assays where microwaves were used to terminate the reaction before the absorbance was measured in a microtitre plate reader (Electronic Corporation of India, Hyderabad). Thus, this approach, of terminating the assay reaction by exposing it to microwaves, allowed a two-step assay to be used to measure lipase activity. Calculating the enzyme activity shows that the percentage error in calculated activity for three independent experiments (carried out in triplicate) is ±2%. Gupta et al [7] showed that the average measurement error for lipase activity using their method for different lipases was in the range of ±2.1–17%.

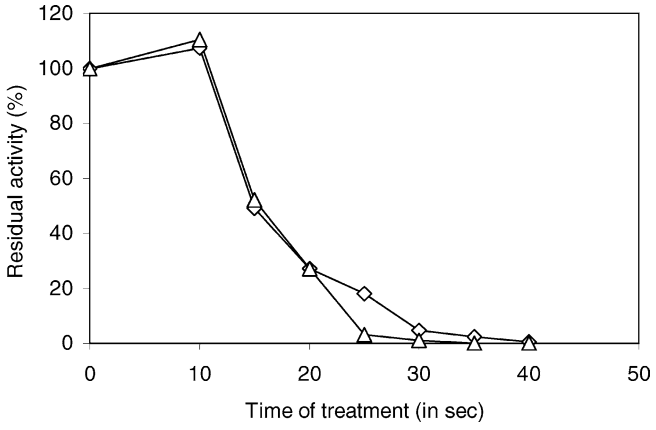

Fig. 2 Effect of microwave treatment of lipase on its esterolytic activity. Lipase (1 and 2 µg) was microwave-irradiated for different time intervals and its activity was checked: *triangles* 1 µg, *squares* 2 µg

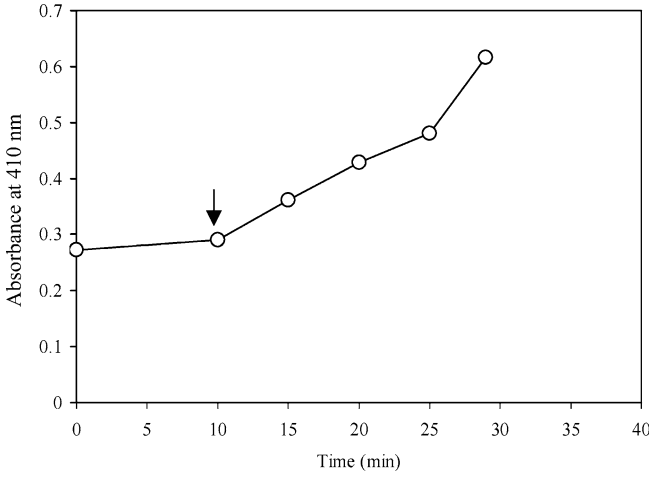

Fig. 1 Change in absorbance of reaction mixture (enzyme + substrate solution) with time. The reading at 0 min shows the absorbance before the reaction is terminated. The *arrow* shows the absorbance of the reaction mixture after cooling it in an ice water bath for 10 min (as the reaction termination step). Subsequent readings after the arrow refer to changes in absorbance with time when the reaction mixture is kept at room temperature

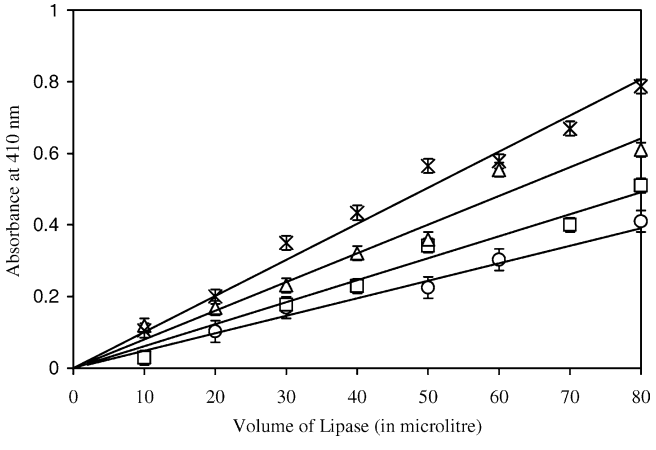

Fig. 3 Standard curves for different lipases in microtitre format. *circles*, *Candida Rugosa* lipase, 0.01 mg/ml; *squares*, *Mucor Javanicus* lipase, 5 mg/ml; *triangles*, *Pseudomonas cepacia*, 0.1 µg/ml; *crosses*, Porcine pancreatic lipase, 10 mg/ml in 0.1 M sodium phosphate buffer pH 7.0. *Error bars* indicate the standard deviation from the mean; each experiment was carried out three times in triplicate. The r^2 values are 0.978 (circles), 0.974 (squares), 0.954 (triangles) and 0.975 (crosses)

Conclusion

The protocol described here is a successful version of a simple and fast assay for lipases. The method should be useful for screening/measuring lipase activity in a large number of samples simultaneously. In view of the biotechnological importance of lipases, lipase assays and screening of lipase activity are integral parts of a large body of research work. Hence, the importance of a convenient assay cannot be overemphasized. Normally, extreme pH conditions or addition of reagents are used to terminate the assay reactions. The idea of using microwaves to terminate a reaction and adopting this technique for use in an assay is a novel one. It should be possible to extend it to other enzyme assays as well. It has been said [10] that microwaves should be used instead of conventional heating to carry out organic transformations. This work indicates that microwaves may have yet another important application: terminating reactions. If a microwave irradiation step can be built-in, the protocol can also be adapted for a high-throughput screening platform.

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