Emulsified Substrate in Flow System: The Determination of Lipase Activity

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Summary: A fast flow system for the quantitative determination of lipase is described. Crude lipase preparation from porcine pancreas is used. The substrate used is *p*-nitrophenyl laurate emulsion in polyvinylalcohol, *p*-nitrophenol is quantitated spectrophotometrically at 405 nm. The detection limit is 0.1 U ml^{-1} and the sample throughput is 25 hr^{-1}. The relative standard deviation for 10 injections of 92.5 U ml^{-1} was 1.5%.

Introduction

Lipases (Triacylglycerol acylhydrolase, EC, 3.1.1.3) have been used as biocatalysts for a variety of reactions, such as hydrolysis of fats, synthesis of glycerides and esters and modification of lipids [1,2]. The high specificity of lipases towards triglyceride substrate with respect to the type and stereospecific position of the fatty acid residue has prompted a number of special applications within the area of food [3-5] and biosurfactants [6,7]. With the increased interest in lipid metabolism, lipase assay in biological fluids is getting attention as a routine test in clinical laboratories [6,9].

Olive oil is a natural substrate for the assay of lipase [8,10], requires much time and is insensitive while the synthetic substrates including laurates of phenols are simple and in general provide fairly sensitive assay systems for lipase.

In the present investigation the procedure has been improved and applied in a flow system to offer the analysis of more sample per unit time with accuracy. The need to develop this fast assay system arose due to the fact that in our laboratory purification of lipases from various sources is in progress and such fast assay procedures play a vital role in purification procedures. The method is based on the hydrolysis of *p*-nitrophenyl laurate by soluble lipase and the rate of formation of *p*-nitrophenol is measured spectrophotometerically at 405 nm using the following reaction scheme.

Results and Discussion

In order to obtain a system with high accuracy, various parameters were studied by univariate approach including reagents concentration, buffers pH values, temperature, flow rate, and mixing coil length.

Preliminary experiments were carried out to find the pH optimum for the activity of soluble enzyme using Tris-HCl buffers (0.05 M) whose pHs ranged from 7.8-9.0. The results are shown in Table 1. Maximum activity is found at pH 8.6. The effect of Barbital buffer (0.05M) was also investigated and a maximum activity was found at pH 8.2. But in the present study, Tris-HCl buffer 0.05 M, pH 8.6 was selected and used subsequently.

Table 1: Effect of variable parameters on the rate of hydrolysis of *p*-nitrophenyl laurate by lipase

Tris-HCl buffer (pH)		7.8	8.2	8.6	9.0	
Absorbance*		0.021	0.024	0.027	0.022	
p-nitrophenyl laurate Concentration (mM)	0.1	0.3	0.6	0.9	1.2	1.5
Absorbance*	0.027	0.038	0.048	0.060	0.048	0.044
Polyvinylacohol (%)		0.01	0.05	0.10	0.015	0.20
Absorbance*		0.20	0.038	0.046	0.053	0.46
Temperature (°C)		20	30	40	50	60
Absorbance*		0.015	0.30	0.043	0.068	0.035

*Mean of three readings.

To select the concentration level of *p*-nitrophenyl laurate for hydrolysis, different concentrations (0.1 - 1.5 mM) of it were used. As shown in Table 1, the peak height absorbance increased gradually upto 0.9 mM and decreased as the concentration was further increased. The *p*-nitrophenyl laurate solution of 0.9 mM was selected for further studies. The effect of emulsifying agent polyvinyl alcohol was also investigated using various

concentrations from 0.01 - 0.2% (w/v). As shown in Table 1, highest absorbance was obtained with 0.15% (w/v) which was selected and used for subsequent investigations.

The temperature dependency of lipase activity on the hydrolysis of *p*-nitrophenyl laurate was investigated using circulating water bath at various temperatures around the mixing coil. The results are shown in Table 1. There is an increase in response with increase in temperature upto 50 °C and on further increase in temperature the hydrolysis of *p*-nitrophenyl laurate was decreased. The temperature of the mixing coil was maintained at 30 °C for further optimization.

The effect of flow rate and mixing coil length was also investigated in order to obtain the best overall response of the system in terms of sensitivity and rapidity. The stream of emulsion gave a significant response at a flow rate of 0.1 ml min^{-1} while the stream of Tris-HCl Buffer (0.05 M, pH 8.6) gave maximum absorbance at a flow rate from 0.1 ml $min.^{-1}$ with gradual decrease in absorbance to 0.32 ml min^{-1} having effect on sample dispersion, nonsteady baseline, dual peak response and longtime consumption (6-12 samples hr^{-1}) and therefore a flow rate of 0.64 ml min^{-1} was used throughout the experiment which gave a steady baseline and single sharp peak. There was a slight gradual increase in peak height absorbance when the mixing coil length was increased from 90 - 440 cm. A mixing coil length of 340 cm was found to be suitable and therefore was selected. The effect of sample loop was also optimized and a sample loop of 90 µl was chosen for all further studies.

Analytical performance

A stock solution of lipase (triacylglycerol acylhydrolase: EC, 3.1.1.3. from Rugosa pseudomonas; Sigma) with an activity of 44 U mg^{-1} was prepared by disssolving 0.7 mg in 1.4 ml of Tris-HCl buffer (0.05 M, pH 8.6). From this stock solution a series of standard solutions covering the range 1.1, 2.2, 3.3, 4.4 and 5.5 U, diluted upto 1 ml with Tris-HCl buffer (0.05 M, pH 8.6) were injected using optimised conditions. The calibration data is given in Table 2. The detection limit (twice to blank noise) was 0.1 U ml^{-1}. The sampling rate was 25 hr^{-1} and the r.s.d. for 10 injections of 92.5 U ml^{-1} was 1.5%.

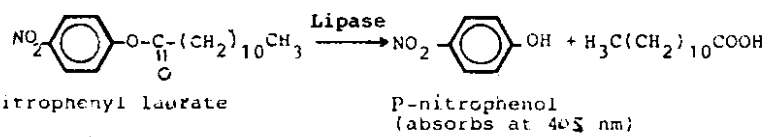
Table 2: Calibration data for lipase

Concentration (U ml^{-1})	1.1	2.2	3.3	4.4	5.5
Absorbance*	0.016	0.033	0.045	0.057	0.066
R.S.D (%)	0.7	0.4	1.3	1.7	2.3

*Mean or three readings.

Conclusion

The FIA system reported has a reasonable sample throughput, flexible and can easily be adopted for the analysis of biological samples. The sensitivity of the assay can be increased by stopping the enzyme with the reagent in the mixing coil for 2-4 min. but the sampling rate will be decreased.



Experimental

Crude lipase preparation (type II) from porcine pancreas was purchased from Sigma. The crude enzyme powder 100 mg was suspended in 40 ml of deionised water and a clear suernatant was obtained by centrifugation at 5000 rpm for 30 min., stored at 4 °C and used whenever required. *p*-Nitrophenyl laurate from Sigma, polyvinyl alcohol from Fluka were purchased. All other chemicals used were AnalaR grade.

Emulsion preparation

A stock solution (20 mM) of *p*-nitrophenyl laurate was prepared by dissolving 0.0642 g in acetone. From this stock solution 0.9 ml was added to

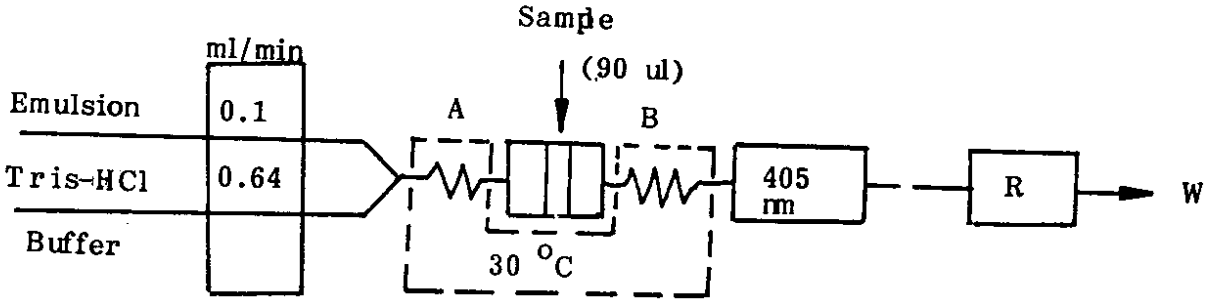


Fig. 1: Dual channel FIA manifold for lipase deter mination. A = Mixing coil 30 cm, B = Mixing coil 340 cm. R = Recorder, W = Waste

a mixture of 6 ml of acetate buffer (0.05 M, 4.0 optimized) and 3 ml of 1% aqueous polyvinyl alcohol solution and emulsified. The emulsion was diluted with deionised water to a total volume of 20 ml. The emulsion is stable for three days when stored at 4 °C and therefore, fresh emulsion was prepared whenever required.

The instrumentation used in the prepsent work is shown Fig. 1 which consists of Ismatec (Reglo 100) peristaltic pump, Model 5020 Rheodyne injection value equipped with a 90 µl loop and a spectrophotometer (LKB, Novaspec II) with a flow through cell (30 µl) connected to a chart recorder (Kipp & Zonen, BD 40). Standard size teflon tubings (0.5 mm, i.d.) were used to connect the components of flow injection system.

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