

Standardization of reaction kinetics for enzymatic hydrolysis of Indian mustard seed oil for extraction of Erucic acid

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Abstract

Erucic acid (EA) constitutes about 30-51% of the total fatty acids of Indian mustard (*Brassica juncea* L. Czern. & Coss.) seed oil. It has got many industrial applications. Hydrolysis of mustard oil by commercial lipase enzyme, Lipozyme TL-100L (source: Novozymes) was investigated for the extraction of EA. The free fatty acids (FFA) present in the mustard seed oil were removed by repeated washing with 90% aqueous ethanol before performing hydrolysis. Hydrolysis was performed at a temperature of 55 °C and pH of 5-6 in a water bath shaker for different enzyme concentrations (0.01, 0.02, 0.5, 1.0 and 3.0%) and time periods (30min, 1hr, 3hr, 6hr and 24hr). Released fatty acids were extracted with NaHCO₃. This was simple, easy and gave comparatively more recovery of EA. Percentage of FFA as oleic analyzed for different hydrolysis time intervals of 1hr, 2hr, 3hr, 4hr, 5hr, 6hr and 24hr and was minimum at 0.3% and maximum at 0.01% as 1.17 and 4.32 respectively for a time period of 24hr. Percentage of EA in the released acid fraction was analyzed by Gas Chromatography. The analysis revealed that EA content showed a reduction with an increase in the concentration of lipase enzyme used for hydrolysis after threshold equilibrium. EA content was found to be 81.50% (30min); 84.30% (1hr); 90.05% (3hr); 99.30% (6hr); 88.63% (24hr) for 0.01% and 79.59% (30min); 86.49% (1hr); 82.56% (3hr); 80.01% (6hr); 69.43% (24hr) for an enzyme concentration of 0.05%. As lipase, TL-100L is 1, 3 specific therefore with more concentration of enzyme the percentage of EA hydrolysis was decreasing as it started hydrolyzing other relevant positions in triglyceride chain after a certain time period and percentage. It was observed that enzyme concentration of 0.01% for a period of 6hr was the ideal parameter for enzymatic erucic acid extraction from mustard oil.

Key words: Erucic acid, lipase, mustard seed oil, hydrolysis

Introduction

Erucic acid (EA) is 22-carbon monosaturated fatty acid with a single double bond at the omega 9 position. EA constitutes about 30-51% of the total fatty acids of Indian mustard (Kaushik and Agnihotri, 2000). EA has various industrial applications from lubricants to slip agent in plastic industry. The primary use of EA is a precursor to another chemical erucamide. Erucamide is used in the processing of plastic film, particularly for film made from polymers, polyethylene and polypropylene. The market for erucamide along with other important uses for EA such as personal care products and lubricants derives an annual world demand to a great extent. Therefore EA extracted from mustard oil will be an alternative to many petrochemical products. Till now high pressure fat splitting of mustard oil has remained in practice but recently with the advancement in the field of biotechnology this focus has shifted towards the 'Green chemistry', i.e. biocatalysts. The enzyme-mediated hydrolysis is an alternative to tedious and expensive high-pressure methods.

Lipases, triacyl glycerol hydrolases are an important group of biotechnologically relevant enzyme and their applications in several areas of biotechnology has been well studied (Gupta et al., 2004). This group of enzymes can catalyze the hydrolysis of fatty acid ester bond in triacyl glycerol thus releasing FFA under aqueous and non-aqueous conditions as well. Therefore, the enzyme lipases are the best fit enzymes for the hydrolysis of mustard oil. The role of lipase in lipid metabolism is widely studied (Gordillo et al., 1998; Knezevic, 2004; Gomez et al., 2005; You and Baharin, 2006; Srivastava et al., 2006). Lipase hydrolysis of mustard oil under water limited conditions yield dierucin, which can be easily isolated (Kaimal et al., 1993). EA forms 25.64% of the total fatty acid composition in a study of mustard seed oil by commercial lipase from *Candida rugosa* (Tinoi, 1999). EA content in the FFA reaction reached 82.2% with a recovery of 71.7% during enzymatic hydrolysis of Crambe oil using porcine pancreatic lipase (Tao and He, 2005). Partial hydrolysis of triacyl glycerols of high EA seed oils catalyzed by lipases from *Candida cylindracea* and *Geotrichum candidum* leads to enrichment of EA and other very long chain monounsaturated fatty acids in the acylglycerols while the C₁₈ fatty acids are enriched in the free fatty acid fraction (Mukherjee and Kiewitt, 1996). In this study EA isolation from mustard seed oil was evaluated using enzymatic hydrolysis.

Materials and methods

Commercially refined mustard seed oil was used for the hydrolysis analysis. Lipozyme TL 100 L (Source: *Thermomyces longinosus*) was received from Novozymes A/S (Bagsvaerd, Denmark). The FFA of mustard oil were removed by repeated washing with aqueous ethanol in the ratio of 10:90 before hydrolysis. The enzyme concentrations used were 0.01, 0.02, 0.5, 1.0 and 3.0%. The time parameters were 30min, 1 hr, 3hr, 6hr and 24 hr. pH of the reaction medium was maintained at 5.5. Hydrolysis was performed at 55°C in a water bath shaker. Solvents were either of HPLC grade or AR grade.

Analysis of % oil hydrolysis: Percent oil hydrolysis was estimated titrimetrically by comparing the alkali (0.1N KOH) used to neutralize the released fatty acids. The results were expressed as percentage of FFA as oleic acid = $28.2x N \times V / W, N$

= Normality of KOH solution, V = Volume of KOH solution used (ml), W = Weight of the sample (gm).

Extraction of EA: EA was isolated from the hydrolysis mixture through two different approaches. viz. Solvent extraction using aqueous ethanol and NaHCO_3 extraction.

1) *Solvent extraction using aqueous ethanol:* Released fatty acids from mustard oil as a result of enzymatic hydrolysis were extracted with solvent extraction approach. Aqueous ethanol in the ratio of 30:70-10:90 was used to isolate EA. Separation was carried out by separating funnel. After thorough washing with 90% aqueous ethanol, the ethanol extract was evaporated and used for fatty acids profile estimation.

2) *NaHCO₃ extraction:* At the completion of reaction, NaHCO_3 was added for increasing the pH and for transferring the released fatty acids in to the aqueous phase. It was followed by adding 1 volume of fat solvent. Resultant was centrifuged to 10,000 g to pellet out the fatty acid salt and to remove the oil. Pellet was washed twice with fat solvent. After evaporating the fat solvent pellet was resuspended in 10 ml of distilled water and its pH was adjusted to 2 with the help of 0.1 N HCl and 2 volumes of fat solvent was collected and was concentrated by evaporation.

Estimation of hydrolysis products: Fatty acids estimation was done with the help of Gas Liquid Chromatography (GLC). Fatty acids were converted to their respective methyl esters by using 0.5 N methanolic potassium hydroxide before GLC analysis in heptane as solvent medium.

Fatty acid esterification: 1 ml of 0.5 N methanolic potassium hydroxide was added to 15 ml fatty acids solution dissolved in n-heptane and was vigorously shaken for 5 minutes and was kept stand by for next 5 minutes for phase separation. Heptane layer was collected and two washes with distilled water were given to remove the traces of KOH from it. Heptane was evaporated to 1/5th volume at 70^o C.

GLC analysis: GLC analysis of fatty acid methyl ester was done at oven temperature of 130^oC and column temperature 230^oC with FID having nitrogen as a carrier at a pressure of 4 kg per cm² and 0.2 μl of methyl ester was injected for analysis.

Statistical analysis: The experiments were performed in ten replicates and the statistical analysis was done using the ANOVA test in the Stat plus package.

Results

Hydrolysis of mustard seed oil with lipozyme TL 100 L at various time periods and enzyme substrate ratios have been depicted in the Fig.1. Kinetics of mustard oil hydrolysis was performed for different enzyme concentrations (0.01, 0.02, 0.5, 1.0 and 3.0%) and for the reaction time periods of 1, 2, 3, 4, 5, 6 & 24 hr. The percentage of FFA had increased from 5.85 to 10.46, when the amount of enzyme used was 0.01% and 3.0% respectively for a reaction period of 5hr. Percentage of FFA released after hydrolysis was maximum at an enzyme concentration of 0.01% and minimum at 3% i.e. 4.32 and 1.17 respectively for a time period of 24hr. Isolation of EA from the fatty acids released as a result of enzymatic hydrolysis of mustard seed oil was done using two extraction methods. Extraction methods were analyzed for different enzyme concentrations (0.01, 0.02, 0.5, 1.0, 3.0%) and reaction time periods (30 min, 1, 3, 6 & 24 hr). EA content in the free fatty acids fraction reached to 93.21% and 99.30% after a reaction time period of 6hr and 46.00%, 88.63% for a reaction time periods of 24hr with an enzyme concentration of 0.01% for solvent extraction and NaHCO_3 precipitation respectively (Fig. 2). Excess enzyme did not contribute to the increase in EA content in the FFA fractions in both the approaches (Table 1 & 2).

Discussion

The extent of hydrolysis of mustard oil by lipozyme was found to be influenced by the enzyme substrate ratios and the reaction time periods. Both the time course and the enzyme concentrations gave an insight into the performance of an enzyme on the reaction progression. It was found that percentage of FFA released was maximum at a reaction period of 5 hr for the entire enzyme concentrations studied. Degree of hydrolysis found to be lower in low enzyme substrate ratio and found to be increasing linearly with an increase in enzyme concentration. There was a significant reduction in the FFA production in the later stages of hydrolysis and it was due to enzyme denaturation by increase in pH with the result the reaction attained equilibrium. During the course of enzyme catalyzed reaction an increase in product concentration can inhibit enzyme activity (Worthing, 1997). EA recovery from released fatty acids was achieved successfully in both extraction methods. But among these approaches NaHCO_3 precipitation is simple, easy and gave comparatively more recovery of fatty acids. Solvent extraction using aqueous ethanol also gave higher percentage of EA in the FFA fraction. Typical solvents used for FFA extraction include, methanol, ethanol and isopropyl alcohol (Kim et al., 1988). Crystallization of EA from the lipase catalyzed hydrolyzed mixture of mustard seed oil was done with aqueous ethanol with 34% purity (Tinoi et al., 2000). But main problems with solvent extraction is its time consuming and tedious process. Through solvent extraction, F.F.A and neutral lipids can not be separated completely (Worthing, 1997). As lipase is considered as 1,3 specific therefore with more concentration of enzyme the percentage of EA is decreasing in the FFA fraction as it starts hydrolyzing other relevant positions in triglyceride chain after a certain time period and percentage. Enzyme concentration of 0.01% for a period of 6hr is working well for both the approaches.

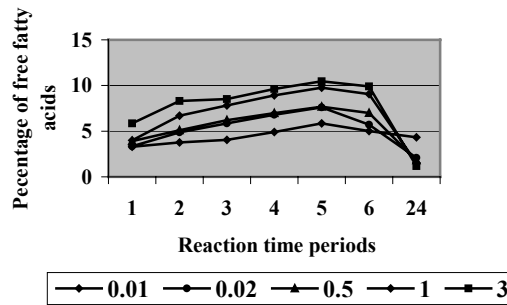


Fig. 1. Hydrolysis course of mustard seed oil with Lipozyme TL 100 L at different reaction time periods (hr) and enzyme concentrations (0.01%, 0.02%, 0.05%, 1.0% & 3%).

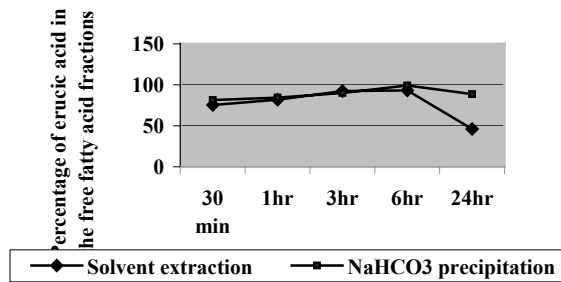


Fig. 2. Percentage of erucic acid in the free fatty acid fractions in different extraction approaches for different time periods. Hydrolysis was performed with an enzyme concentration 0.01% at 55 °C and moisture 25% v/v.

Table 1. Fatty acid composition of mustard oil after hydrolysis for a time period of 6hr at different enzyme concentrations (Extraction method: NaHCO₃ precipitation)

Enzyme concentrations used	Oleic C _{18:1}	Linoleic C _{18:2}	Linolenic C _{18:3}	Erucic C _{22:1}
0.01	0.00	0.00	0.00	99.30
0.02	0.46	0.35	0.35	96.44
0.05	16.61	1.54	0.00	80.01
1.00	5.04	4.01	6.16	76.11
3.00	12.86	12.82	5.34	63.04

Table 2. Fatty acid composition of mustard oil after hydrolysis for a time period of 6hr at different enzyme concentrations (Extraction method: Solvent extraction using aqueous ethanol)

Enzyme concentrations used	Oleic C _{18:1}	Linoleic C _{18:2}	Linolenic C _{18:3}	Erucic C _{22:1}
0.01	0.00	0.24	2.80	93.21
0.02	0.55	0.77	0.74	92.81
0.05	0.83	0.23	3.36	89.53
1.00	1.77	3.24	12.64	75.80
3.00	8.30	3.77	10.12	67.68

Conclusion

Reaction temperature at 55 °C, 25% (v/v) water content for a period of 5 hrs is optimum for the Lipozyme TL 100 L enzymatic hydrolysis of mustard seed oil for the production of FFA. NaHCO₃ precipitation is found to be more suitable extraction method for the recovery of EA than the solvent extraction.

Acknowledgement

This project was funded by Technology Mission on Oilseed, pulses & Maize (TMOP&M), Ministry of Agriculture, India and Council of Scientific and Industrial Research (CSIR), Govt. of India.

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