

속 보

탈지아몬드가루의 Oxynitrilase의 촉매에 의한 Diethyl Malonate의 Aldehyde에 첨가

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The Addition of Diethyl Malonate into Aldehydes Catalyzed by Oxynitrilase in Defatted Almond Meal

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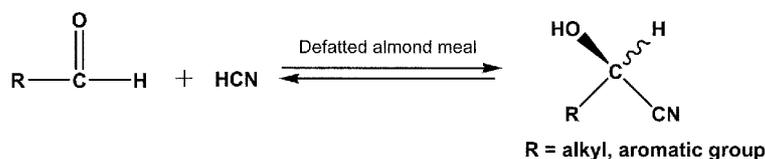
Defatted almond meal, which contains oxynitrilase, has been used as a catalyst for the production of aromatic and aliphatic (R)-cyanohydrins. Oxynitrilase, which is also known as mandelonitrile lyase (or hydroxynitrile lyase, Hnls), catalyzes the reversible formation of cyanohydrins from HCN and aldehydes or ketones (Scheme 1).¹ The reverse reaction occurs because HCN acts as a defense system against microbial attack.²

In 1908, oxynitrilase was used as a catalyst in asymmetric synthesis.³ Now more than 3000 plants are known to contain this enzyme.^{4,5} Oxynitrilase have received considerable attention in industry for the synthesis of chiral compounds, which are formed from the stereospecific addition of HCN to aldehydes or ketones.⁶ This enzyme contains a broad range of substrates ranging from aliphatic, aromatic aldehydes and ketones as HCN acceptors. There has been less focus on the changes in HCN in the oxynitrilase reaction. Therefore, there is limited information on the use of other substrates instead of HCN. Enzyme promiscuity has become a new field in biocatalysis,⁷ and concerns the ability of an enzyme to catalyze more than one type of chemical modifica-

tion. In an oxynitrilase biotransformation, the molecular size and pKa of the acidic portion (HCN, pKa=9) are critical parameters.⁸ Based on these criteria, nitromethane and nitroethane were used instead of HCN to react with various aldehydes in the presence of oxynitrilase purified from *Hevea brasiliensis* (HbHNL).⁹

Diethyl malonate was used in oxynitrilase reaction instead of HCN. This paper reports a systematic study on the introduction of diethyl malonate into aldehydes with oxynitrilase in almond meal. 1,3-Dicarbonyl compounds are widely used as C-nucleophiles in the Michael addition.¹⁰ The pKa of diethyl malonate is 13 but the molecular size of diethyl malonate is larger than that of nitroethane. In the asymmetric hydrocyanation using (R)-oxynitrilase from almond, the isolated and purified enzyme from the almond meal did not have much effect on the optical purity of product.¹¹ Therefore, an attempt was made to introduce diethyl malonate into aldehydes using the enzyme in defatted almond meal without purification.

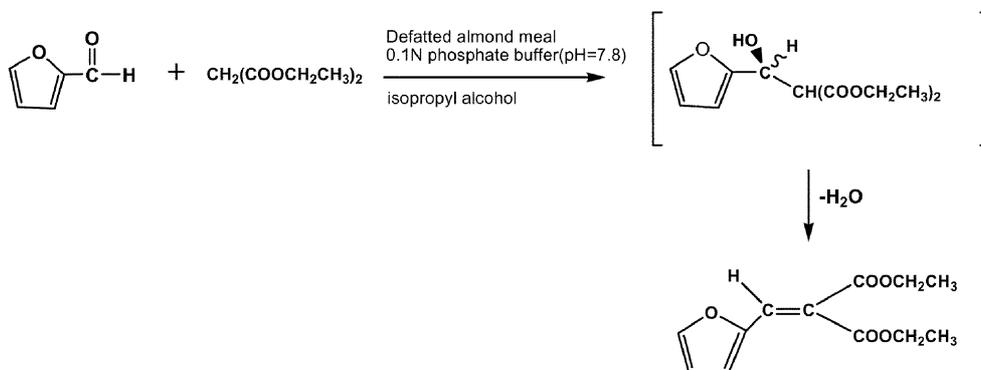
HCN is added to the aldehyde chemically when acetone cyanohydrin is used as the substrate. Ini-



Scheme 1.

tially, the conditions where reaction would not occur without the enzyme were examined. It was found that aldehyde and diethyl malonate in a mixture of a phosphate buffer and organic solvent at 28 °C does not lead to any product formation after 48 hours. Toluene and isopropyl alcohol were used as the organic solvents to monitor the chemical addition. We were pleased to find that the reaction provided no desired product in the absence of defatted almond meal.

The almond was ground in a mortar and washed with ethyl acetate until the filtrate was colorless. The defatted almond meal was stored at 5 °C. The addition of diethyl malonate to aldehydes in the presence of defatted almond meal was examined. As expected, oxynitrilase in defatted almond meal accepted diethyl malonate as a substrate. Unfortunately, the alcohol intermediate that formed from the condensation of the aldehyde and diethyl malonate was dehydrated. A reaction of 2-furaldehyde with diethyl malonate gave the dehydrated product instead of the alcohol product (Scheme 2). In the bio-catalytic Henry reaction, Gruber *et al.* reported that 10–15% of the elimination product, 1-nitro-2-phenyl ethene, was produced as a by-product.⁹



Scheme 2.

Table 1. Effect of substrate structure on the enzymatic reaction

$$\begin{array}{c}
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 \parallel \\
 \text{R}-\text{C}-\text{H} + \text{CH}_2(\text{COOCH}_2\text{CH}_3)_2 \xrightarrow[\text{iso propyl alcohol}]{\text{Defatted almond meal, 0.1N phosphate buffer (pH=7.8)}} \begin{array}{c} \text{H} \\ \diagdown \\ \text{C} \\ \diagup \\ \text{R} \quad \text{C} \\ \diagdown \quad \diagup \\ \text{COOCH}_2\text{CH}_3 \quad \text{COOCH}_2\text{CH}_3 \end{array}
 \end{array}$$

Entry	R	Product	Yield (%)
1		1	30
2		2	58
3		3	64
4		4	4
5	CH ₃ (CH ₂) ₅	5	46

Diethyl methylmalonate was used to prevent the dehydration of the alcohol intermediate. However, there was no reaction (Table 3). The reason for this is probably the limited access to the active site of the enzyme.¹² The pKa of diethyl methylmalonate is different from that of diethyl malonate and steric hindrance of the methyl group would be another factor. p-Anisaldehyde was found to be poor substrate for the enzyme, converting very little of the

Table 2. Effects of buffer pH on oxynitrilase in defatted almond meal catalyzed synthesis of **3**.

Entry	pH	Yield (%)
1	5.8	5
2	6.6	22
3	7.2	52
4	7.8	64
5	8.0	60

Table 3. Addition of malonate derivatives to 2-furaldehyde in the presence of defatted almond meal

Entry	substrates	Yield (%)
1	diethyl malonate	64
2	diethyl methylmalonate	0

compound to product (4% yield). The electron-donating group at the 4' position of the phenyl ring decreased the reaction rate. In general, a change of aldehyde does not alter the product yield except p-anisaldehyde.

Oxynitrilase shows remarkable substrate scope in the asymmetric cyanation of various aldehydes and ketones. Diethyl malonate was found to be a good substrate even though hydrogen cyanide is the original substrate in oxynitrilase. The dehydration of alcohol intermediate occurred during the enzymatic reaction. The dehydration product, 1-nitro-2-phenyl ethene, was obtained during the enzymatic introduction of methyl nitrate into aldehydes.⁹ The enzyme has a typical catalytic triad and serine (Ser₈₀) and histidine (His₂₃₅) residues are essential for its activity.¹³ Some residues in the active site of the enzyme would participate in the dehydration process. These results show that only the dehydrated products were obtained. One of the reasons for this is that the final product was highly conjugated. The dehydrated product is more thermodynamically stable, and the alcohol intermediate might be a favorable position for dehydration in the enzyme active site.

The buffer pH strongly influences the enzymatic activity. The pH-dependence of the enzymatic introduction of diethyl malonate was examined over a pH range of 5.8-8. Generally the pH range of almond meal as source of oxynitrilase ranges from 5.5 to 8.0 with the best conversion yield being obtained at

Table 4. Effects of organic solvent and temperature on hydroxynitrile lyase in defatted almond meal catalyzed synthesis of **3**.

Entry	Solvents	Temp. (°C)	Yield (%)
1	isopropyl alcohol	28	64
2	isopropyl alcohol	5	30
3	isopropyl ether	28	11
4	toluene	28	6

pH=7.8, as shown in Table 2. Diisopropyl ether, isopropyl alcohol and toluene were used as cosolvents for the enzymatic reaction. As shown in Table 4, a higher yield was obtained in a more hydrophilic solvent. This can be explained by the higher concentration of diethyl malonate in the buffer solution when a more hydrophilic solvent is used.

In conclusion, substrates other than HCN were used in the oxynitrilase reaction. By using defatted almond meal, diethyl malonate was introduced to various aldehydes. Introduction of diethyl malonate to aldehydes proved to be quite difficult, which was partly attributed to the low reactivity of diethyl malonate and the higher steric hindrance. It should be noted that the results of this initial screening work were obtained with minimal process development. Further refinement of this biotransformation will involve determining the mode of substrate binding to enzyme.

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14. General procedure: 2-Furaldehyde (500 mg, 5.2 mmol) and diethyl malonate (916 mg, 5.7 mmol) was added to a solution of defatted almond meal (500 mg) in phosphate buffer (0.1 M, pH=7.8, 15 ml) and isopropyl alcohol (15 ml). The reaction mixture was stirred for 48 hrs at 28 °C. The reaction mixture was filtered through a celite pad and the isopropyl alcohol was evaporated. The aqueous layer was extracted with ethyl acetate (15 ml × 2). The combined organic layers were dried over MgSO₄ (anhydrous) and concentrated. The crude reaction mixture was purified by silica gel column chromatography. **3**(liquid, 872 mg, 64%). ¹H NMR (500 MHz, CDCl₃): δ 7.5(s, 1H), 7.4(s, 1H), 6.7(d, *J* = 3.4 Hz, 1H), 6.4(m, 1H), 4.4(q, *J* = 7.4 Hz, 2H), 4.2(q, *J* = 7.2 Hz, 2H), 1.3(m, 6H).
15. ¹H NMR (500 MHz, CDCl₃): **1**. 7.7(s, 1H), 7.4(m, 2H), 7.3(m, 3H), 4.3(m, 4H), 1.3(t, *J* = 7.1 Hz, 3H), 1.2(t, *J* = 7.1 Hz, 3H), **2**. 7.8(s, 1H), 7.5(d, *J* = 4.8 Hz, 1H), 7.3(d, *J* = 3.4 Hz, 1H), 7.0(m, 1H), 4.6-4.2(m, 4H), 1.5-1.2(m, 6H), **3**. 7.6(s, 1H), 7.4(d, *J* = 8.8 Hz, 2H), 6.9(d, *J* = 8.8 Hz, 2H), 4.3(m, 4H), 3.8(s, 3H), 1.3(m, 6H), **4**. 6.9(t, *J* = 7.8 Hz, 1H), 4.3(m, 4H), 2.3(q, *J* = 7.4 Hz, 2H), 1.4-1.2(m, 14H), 0.8(m, 3H).