行政院國家科學委員會專題研究計畫成果報告

石蓴之鈣活化脂氧合酶之固定化與活化機制

(Immobilization and Activation Mechanism of Calcium Stimulated Lipoxygenase from Ulva)

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一、中文摘要

本研究乃探討石蓴脂氧合酶經固定化後之特性。石蓴粗抽出液經 40-55％硫銨分劃、MacroPrep-Q 離子交換及 Sephacryl S-300 膠過濾後，脂氧合酶活性(LOX)純化了 103 倍。八種不同抑制劑對脂氧合酶之影響以 NDGA, BHA, esculetin, $SnCl_2$ 之抑制效果最高，脂氧合酶之催化機制似乎與其構造或自由基之產生有相當大之關係。於幾種固定化方法中以 Chitosan-carbodiimide-glutaraldehyde 之固定化方法效果較好。最適之 glutaraldehyde 或 carbodiimide 之用量分別為 0.03 及 0.04％。經固定化後其最適溫度提高了約 7℃左右，最適 pH 不變；對熱、pH 之安定性皆提高。經固定化後之 LOX 對溫度之耐性較未經固定化者高出甚多。固定化之海藻 LOX 於高溫下之操作穩定性較差，而於室溫或 4℃下之操作穩定性較高。

關鍵詞：*脂氧合酶、海藻、石蓴、純化、固定化酵素。*

Abstract

A calcium stimulated-lipoxygenase (LOX) was isolated and purified 103-fold from sea algae (*Ulva lactuca)* using 40-55% saturation of ammonium sulfate fractionation, MacroPrep-Q ion exchange, and gel filtration on Sephacryl S-300. Eight synthetic LOX inhibitors including BHA, BHT, PG, esculetin, esculin, NDGA, $SnCl_2$, and $HgCl_2$ were used to study the activation mechanism of this partially purified LOX. Among them, NDGA showed the highest inhibition, followed by BHA, esculetin, and $SnCl_2$. It seems that the conformation change and the formation of free radical were involved in the activation process of algal LOX. The partially purified algal LOX was then immobilized with Chitosan-carbodiimide-glutaraldehyde (CN-EDC-GA) system, and the optimal condition for the immobilization was listed in the following: glutaraldehyde, 0.03%, carbodiimide, 0.04%. The optimal temperature of the immobilized algal LOX was 7℃ higher than that of soluble form, while the optimal pH was the same with soluble LOX. Moreover, the immobilized algal LOX exhibited higher pH stability and thermostability than those of soluble LOX. The immobilized algal LOX displayed the best operation stability with a denaturation half life of 360 *h* at 4 ℃; 195 *h* at 26℃.. In conclusion, the immobilization of algal LOX on CN-EDC-GA system greatly enhanced the stability of the enzyme against thermal denaturation.

Keywords: ***Lipoxygenase, sea algae, Ulva lactuca, purification, immobilization***

二、緣由與目的

水產香料為高單價產品，例如蝦蟹香料每公斤可高達兩千元以上。市售之水產香料多由國外進口，目前尚無民間企業自行生產者，故水產香料之研究與開發實有其必要性。市售水產香料之香氣物質較不足，如何強化此等水產香料使其更接近海鮮香氣，仍值得研究。為強化市售水產香料之香氣，探討具水產或海鮮香氣之化合物或形成機制，可能

是未來此一領域之研究發展的重點。(Pan and Kuo, 1994)

生鮮魚味之香氣成分可由不飽和脂肪酸經脂氧合酶與過氧化物水解酶催化作用產生。脂氧合酶若被抑制，則鮮魚味之揮發性成分幾乎無法形成。植物香氣之形成亦與脂氧合酶有關，植物成熟時散發之香氣成分，主要與脂氧合酶與過氧化物水解酶代謝脂質有關。魚漿中加入植物之脂氧合酶會產生強烈之鮮魚味 。 以脂氧合酶處理 22:6 後，於微波加熱過程形成柴魚味。烏魚鰓或海藻之脂氧合酶亦被應用於修飾魚油(胡，1998)或烏魚子味道或形成水產香氣。除此之外，脂氧合酶亦有改善蝦香氣之效果(Kuo and Pan, 1991, 1992, Pan and Kuo, 1994, Kuo, et al., 1994, 1996 a & b, 1997).。

於84、85、86年度計畫 "海藻脂氧合酶之純化及對水產香氣形成之影響" (NSC-84-2214-E-041-002、NSC 85-2214-E-041-001、NSC 86-2214-E-041-001)之研究中發現兩種綠藻，石髮(*Enteromorpha intestinalis*)與石蓴(*Ulva lactuca*) 之脂氧合酶活性極高。石髮與石蓴之脂氧合酶已部分純化，並已確定其最適反應條件、催化性質及基質特異性(Kuo, et al., 1996 a & b, 1997)。生鮮石髮有牡蠣香氣，經微波加熱後則有海螺香氣； 利用 GC-sniffing 分析石髮抽出物中有具甜瓜味、青蘋果、芒果青及海苔等香氣成份 。將海藻脂氧合酶抽出液添加入魚漿、蝦頭殼或魚油中確有改善風味之效果，並使其中之風味物質大幅增加(胡，1998)。然而魚鰓、海藻之脂氧合酶不十分安定，並有自殺性失活現象(Kuo, et al., 1994, 1996 a & b, 1997).，因此如何使脂氧合酶安定為利用該酵素之首要課題。

本計畫中將研究石蓴脂氧合酶之特性並利用固定化酵素方法探討穩定脂氧合酶活性之可行性。藉海藻脂氧合酶形成水產香氣，將可提高海藻之利用價值。由水產香氣物質形成之途徑及最適條件，或可作為未來發展水產香料之參考。

三、結果與討論

石蓴粗抽出液經 40-55％硫銨分劃、MacroPrep-Q 離子交換及 Sephacryl S-300 膠過濾後，脂氧合酶活性(LOX)純化了 103 倍，回收率為 33％（表 1）。該部份純化酵素之最適 pH 及溫度、對於不同脂肪酸或其酯類衍生物之反應性、催化脂肪酸之氧化產物以及受鈣活化之現象已於另文探討(Kuo, et al., 1997)。為進一步了解其活化機制，使用 8 種不同抑制劑探討活性之消長情形如表二所示。其中以 NDGA, BHA, esculetin, $SnCl_2$ 之抑制效果最高，BHA 為典型之自由基捕捉者之 phenolic 抗氧化劑，esculetin 與 NDGA 為具有 catechol 構造，極易接近脂氧合酶之 active site，而 $SnCl_2$ 則為脂氧合酶特異性抑制劑，似乎脂氧合酶之催化機制與其構形或自由基之產生有相當大之關係。

因脂氧合酶具有自殺性失活之現象，影響其利用性甚巨。故將 LOX 經過硫銨分劃以去除大部分葉綠素，利用固定化技術嘗試穩定其活性。於幾種固定化方法中發現利用 Chitosan-carbodiimide-glutaraldehyde 之固定化方法效果較好。最適固定化條件之探討如表一及表二所示，glutaraldehyde 或 carbodiimide 之最適用量分別為 0.03 及 0.04％，此兩者為後續實驗採用之條件。經固定化後之海藻 LOX 與未經固定化者之最適作用溫度、pH 及熱安定性、pH 穩定性，如表三、四所示。經固定化後其最適溫度提高了約 7℃左右，最適 pH 不變；對熱、pH 之安定性皆提高。進一步將時間延長，以探討較高溫度對固定化後海藻 LOX 之影響，結果如圖 5 所示。經固定化後對溫度之耐性較未經固定化者高出甚多。

為仿造工業應用時之條件，於不同溫度震盪保溫下，每隔 1-6 小時以抽氣過濾更換基質溶液，以測定固定化海藻 LOX 之操作穩定性，結果如表三所示。高溫下海藻 LOX 之操作穩定性較差，而於室溫或 4℃下之操作穩定性較高。由於海藻 LOX 於室溫或 4℃下即具有甚高之反應性，故於日後工業之應用上，使用室溫或 4℃即可，無須更高溫度。

四、謝辭

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六、自評

本研究係有關固定化海藻脂氧合酶之首篇報告，並已順利完成計畫之目標。由於石蓴之風味佳且其 LOX 活性也高，故甚具發展潛力。然而過去在利用脂氧合酶時常遭遇其自殺性之失活問題，影響其利用性甚巨。於本研究中發現 LOX 經固定化後其穩定性增加 10-20 倍以上，將有助於工業上應用此酵素之參考。而後續之原因及機制探討仍有待進一步研究。

Table.1 Purification of lipoxygenase from sea algae (*Ulva lactuca*).

stage	total activity (μmole/min)	total protein (mg)	specific activity (μmole/mg-min)	recovery (%)	purification (fold)
crude extract	97.60	521.4	0.19	100	1.00
40-55 % $(NH_4)SO_4$	64.46	149.2	0.43	66.04	2.31
MacroPrep-Q	37.91	8.2	4.64	38.84	24.79
Sephacryl S-300	32.25	1.7	20.41	33.04	102.66

Table.2. Effects of various inhibitors on the activity of partially purified algal lipoxygenase [a].

inhibitor	IC_{50} (μM)	oxidation potential[C] (volts)
BHA	0.45	0.65
BHT	300	0.68
PG (propyl gallate)	50	0.61
esculetin	1.0	0.76
esculin	200	
NDGA [b]	0.3	
$SnCl_2$	1.5	
$HgCl_2$	25	

a: Partially purified algal LOX was preincubated with inhibitor at 26 C for 10 min. then assayed for LOX activity using linoleic acid as substrate.
b: The concentration of inhibitor causing 50 % reduction of enzyme activity.
c: Hsieh et al., 1988.
d: nordihydroguaiaretic acid

Table 3. Operation stability of the immobilized lipoxygenase (LOX) from sea algae (*Ulva lactuca*)..

Temperature (℃)	Denaturation half life of immobilized LOX (*h*) *
4	360.0
26	195.0
37	42.0
40	24.0
45	2.0
50	0.5

*: The reaction mixture containing immobilized algal LOX and linoleic acid in a glass vial was incubating at different temperature on a orbital shaker (300 *rpm*). The substrate was changed by 1-6 h of incubation, and assayed for their LOX activity

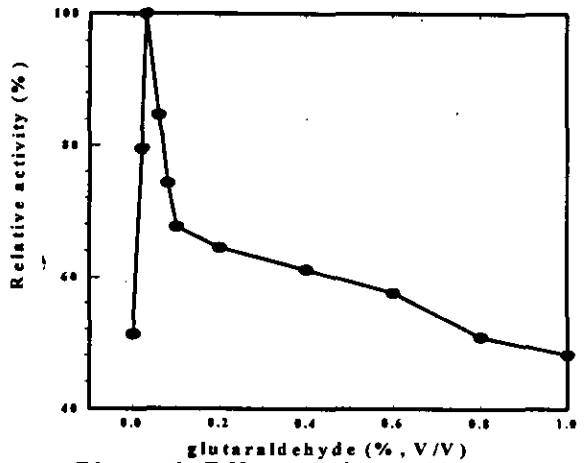

Figure 1. Effect of glutaraldehyde concentration on the relative LOX activity of immobilized algal LOX.

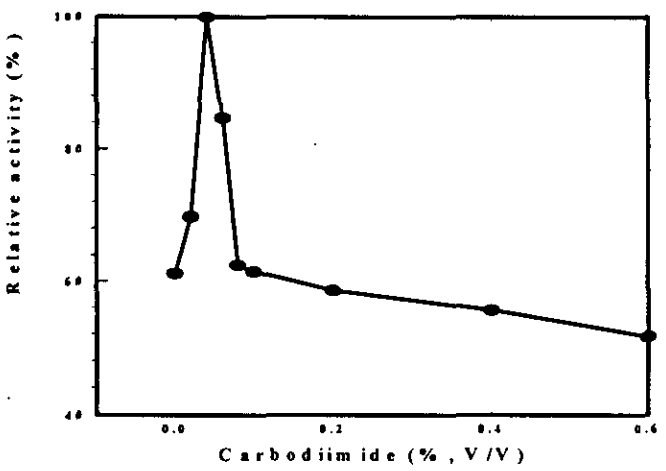

Figure 2. Effect of carbodiimide concentration on the relative LOX activity of immobilized algal LOX.

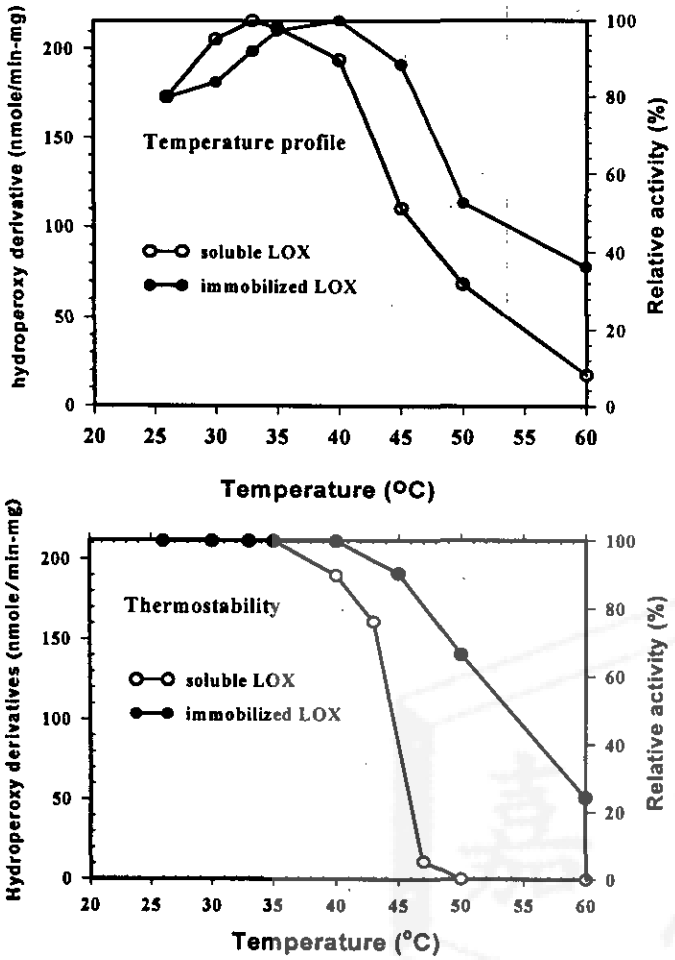

Figure 3. Temperature profile and thermostability of LOX activity of soluble and immobilized enzyme from sea algae. Linoleic acid was used as substrate and reacted at different temperature for 10 min. The thermostability was performed by preincubating algal LOX at different temperature for 10 min and assayed for LOX activity

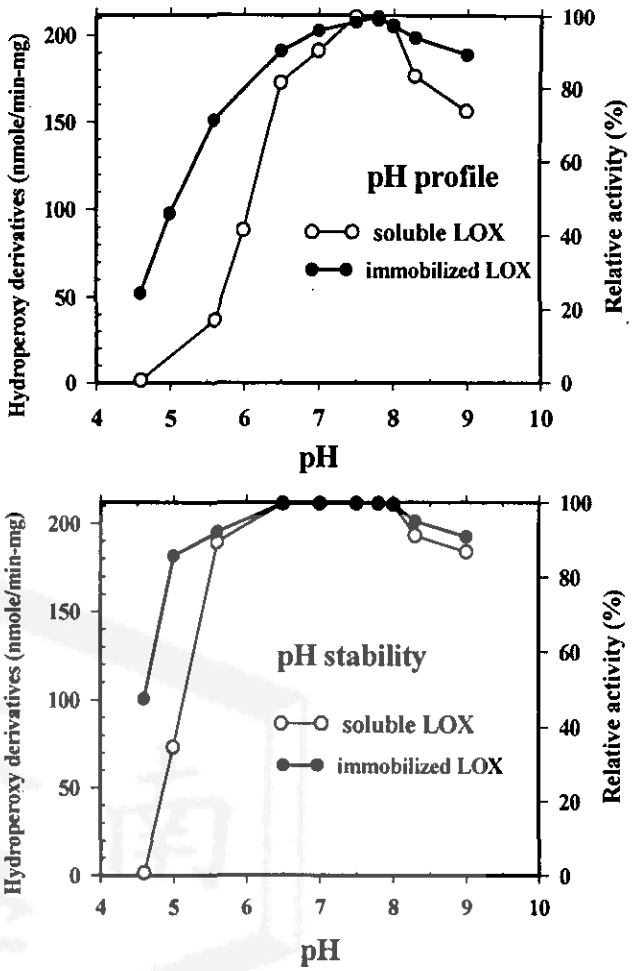

Figure 4. pH profile and pH stability of LOX activity of soluble and immobilized enzyme from sea algae. Linoleic acid was used as substrate and reacted at 26 ℃ for 10 min. The pH stability was performed by preincubating algal LOX at different pH for 10 min and assayed for LOX activity

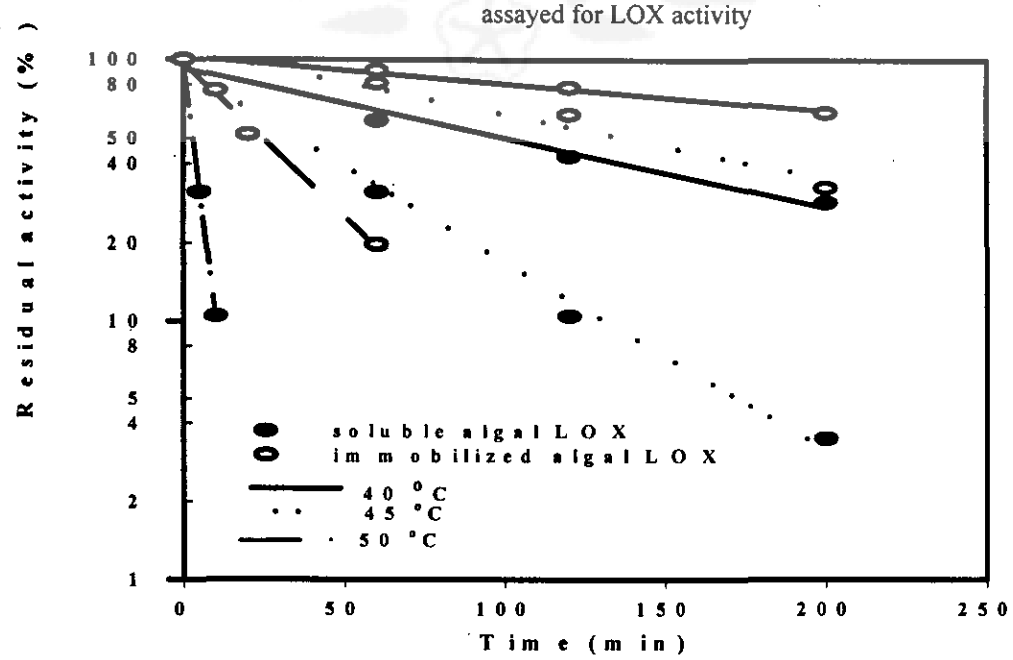

Figure 5. The semilog plot of residual activity vs preincubating time of soluble and immobilized algal LOX at different temperature