行政院國家科學委員會補助專題研究計畫成果報告

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※ *N*-乙醯幾丁寡醣的製備及其對腸內細菌生長的影響 ※
※ Preparation of *N*-acetylchitooligosacchrarides and ※
※ their effect on growth of intestinal bacterial flora ※
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計畫主持人：陳幸臣

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執行單位：國立台灣海洋大學食品科學系

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N-乙醯幾丁寡醣的製備及其對腸內細菌生長的影響

Preparation of *N*-acetylchitooligosacchrarides and their effect on growth of intestinal bacterial flora

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一、中文摘要

以不同聚合度（n =1-6）的 *N*-乙醯幾丁寡醣[$(GlcNAc)_n$]為碳源在培養基中評估 9 株腸內菌的生長情形。此$(GlcNAc)_n$係以鹽酸水解蝦殼的幾丁質製備而得。經過純化後，$(GlcNAc)_{1-6}$的各純度均高於 86%，以這些寡醣或葡萄糖（0.2%, w/v）加入基礎培養基後培養各腸內菌，並以培養期間（48 或 72 小時）之最大細胞濃度（MCD, log CFU ml^{-1}）及比生長速率常數（μ, h^{-1}）評估各菌利用這些糖為主要碳源的生長情形。發現 *Bacteroides fragilis* 及 *Clostridium perfringens* 能有效利用 GlcNAc 及$(GlcNAc)_2$為碳源，而且比利用葡萄糖還好。*Bifidiobacterium adalescentis* 及 *Enbacterium limosum* 只略能利用$(GlcNAc)_{1-6}$。*Escherichia coli*, *Lactococcus lactis* 及 *Proteus vulgaris* 能有效利用葡萄糖，比利用$(GlcNAc)_{1-6}$還好。在$(GlcNAc)_{1-6}$中，GlcNAc 能被 *Staphylococcus aureus* 利用得最好，但卻顯示幾乎相同的比生長速率常數。*Streptococcus faecalis* 在不加糖的基礎培養基中也能生長良好。幾丁質在人類腸道中被降解的可能情形也在本研究中討論。

關鍵詞：*N*-乙醯幾丁寡醣製備、腸內菌、碳源、最大細菌密度、比生長速率常數

Abstract

N-Acetylchitooligosaccharides $[(GlcNAc)_n]$ with different degrees of polymerization (n = 1-6) were prepared as the main carbon sources in the media for evaluating the growth of nine intestinal bacteria. A mixture of $(GlcNAc)_n$ was prepared by hydrolyzing shrimp-shell chitin using HCl. After purification, the purity of each $(GlcNAc)_{1-6}$ was higher than 86%. The growths of intestinal bacteria were carried out in a basal medium containing 0.2% (w/v) of each sugar or glucose as the main carbon source and were evaluated using maximum cell densities and specific growth rates. *Bacteroides fragilis* and *Clostridium perfringens* could more efficiently utilize GlcNAc and $(GlcNAc)_2$, respectively for growth than glucose. *Bifidobacterium adolescentis* and *Eubacterium limosum* could slightly use $(GlcNAc)_{1-6}$ as main carbon source. *Escherichia coli*, *Lactococcus lactis* and *Proteus vulgaris* could more efficiently utilize glucose than $(GlcNac)_{1-6}$. GlcNAc was more availably used than $(GlcNAc)_{2-6}$ by *Staphylococcus aureus*, on revealing almost the same specific growth rates. In basal medium, *Streptococcus faecalis* could grow well even without adding sugars tested. The possible degradation of chitin in human digestive tracts was also discussed.

Keywords: Preparation of *N*-Acetylchitooligosaccharides; Carbon sources; Intestinal bacteria; Maximum cell densities; Specific growth rates

二、緣由與目的

Some of the chitin and chitosan degraded products exhibit bioactivities. Glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) can stimulate proteoglycan biosynthesis. The newly synthesized proteoglycans may stabilize cell membranes, resulting in an anti-inflammatory effect〔1〕. Chitooligosaccharides exhibit various physiological activities including

antimicrobial activity [2,3] , immune-enhancing activity [4,5] , and antitumor activity [6,7] .

The presence of gastric juice in our stomach may degrade shrimp shell to release chitin if the shells are ingested. Chitosan could be formed because of the action of pepsin on chitin in the stomach [8] . Under low pH gastric conditions, some chitin and chitosan may be dissolved. In the small intestine at neutral pH, the chitinous substances would gradually precipitate[9], and moved into the large intestine with slight change. The complexity of the microflora in the colon makes it possible to hydrolyze the chitin and chitosan into GlcN, GlcNAc and their oligomers [10] . Such compounds may affect the growth of intestinal microbiota. Chen and Chen [11] found that the mixture of chitin hydrolysates liquefied by *Cellulomonas flavigena* enhanced the growth of *Bacteroides* in rats ceca. However, the effects of each individual $(GlcNAc)_{1-6}$ on intestinal bacteria growth has rarely been documented. This could be because the oligomers are too expensive for culture media supplementation on a commercial basis. Hydrochloric acid [12] , phosphoric acid [13] , nitrous acid [14] , hydrofluoric acid [15] and formic acid [16] have been employed to carry out the chitinolytic process. Chitinase, lipase, cellulases, hemicellulases, and lysozymes [17-19] have been used to hydrolyze chitin. Due to the enzymatic specificities, to hydrolyze chitin into each individual $(GlcNAc)_{1-6}$ to almost equal amount by a chitinase is impossible so far. This investigation, thus was conducted to prepare a sufficient quantity of each individual $(GlcNAc)_{1-6}$ from chitin hydrolyzed by hydrochloric acid. The prepared substances were then employed as the main carbon sources to evaluate the growth of nine intestinal bacterial strains.

三、結果與討論

The bacterial strains tested in this investigation were selected based on their influences on human health and distributions in human feces. *B. fragilis* can maintain our health, but sometimes it is pathogenic. *C. perfringens, P. vulgaris, S. aureus* and certain strains of *E. coli* are pathogens; while *Bb. adolescentis, L. lactis* and *Sc. faecalis* are probiotic bacteria. *Eu. limosum* is generally regarded as non-pathogenic [21].

N-Acetylglucosamine was more efficiently used than glucose by *B. fragilis*. Salyer et al. [22] found that *B. fragilis* 2393 isolated from human intestine can efficiently utilize D-glucosamine as carbon source. Chen and Chen [11] fed rats on *Cellulomonas flavigena*-degraded chitin hydrolysate [mainly containing $(GlcNAc)_2$ and two unidentified *N*-acetylchitooligosaccharides] and found the distribution of *B. fragilis* increased from 7.8 to 30% after 4-weeks of feeding.

Lactic acid bacteria (*Lactobacillus rhammosus, Lb. plantarum* and *Lactococcus lactis*) can utilize β-gluco-oligosaccharides for growth, but this is reduced according to the increase in the degree of polymerization (2-5). We found that *L. lactis* could utilize $(GlcNAc)_{1-6}$ and revealed almost the same effect. (GlcNAc)1-2 are bifidogenic substances which have not been metabolized by the human digestive system before reaching the colon but can stimulate the growth of bifidobacteria [23] . However in this study, the presence of (GlcNAc)1-2 could not efficiently activate the growth of *Bb. adolescentlis*. This discrepency may be due to the different conditions *in vitro* (culture medium).

A chitin-degrading bacterium *Clostridium* sp. can release $(GlcNAc)_2$ as the major hydrolysis end-product, when chitin is used as a substrate [24] . From our finding that *C. perfringens* could efficiently use $(GlcNAc)_2$ as main carbon source, we infer that the strain may produce exo-*N*-acetylchitobiase.

In Asia, small shrimp like the Taiwan mauxia shrimp (*Acetes erythraens*) and

consumed together with the shells. Because of the action of gastric juice and enzymes in the human stomach and digestive tracts, the shells may finally be hydrolyzed into *N*-acetylchitooligosaccharides, chitooligosaccharides, *N*-acetylglucosamine and/or glucosamine. These amino sugars are not catabolized but rather anabolized into precursors for glycoproteins and muco-polysaccharides formation [9]. Although chitooligosaccharides exhibit antimicrobial activity, this activity is mild as revealed by $(GlcNAc)_{1\text{-}6}$ [2, 3]. Thus, after the ingestion of shrimp shells, the residues from final chitinolytic products may enhance the proliferation of *B. fragilis*, *C. perfringens*, and even *Bb. adolescentis*, since $(GlcNAc)_{1\text{-}2}$ are bifidogenic factors [23]. In our previous study [11], the count of *Bifidobacterium* in the cecal contents of animals fed on chitin hydrolysate was slightly (0.67 log cycle) higher than that in the control $p > 0.05$). However, *C. perfringens* is a pathogen and *B. fragilis* is an opportunitic pathogen, shrimp shell consumption should therefore be clinically studied more thoroughly.

四、計畫成果自評

本計畫進行相當順利。只是在製備相當量的$(GlcNAc)_{1\text{-}6}$時，耗費很多時間。得到$(GlcNAc)_{1\text{-}6}$均勻量的方法，用酸水解是較可行的。若要量產各糖，並以幾丁質酶水解幾丁質以獲得為最可行。這次以 HCl 水解幾丁質，各糖之產率在 3.8~12.6%之間，雖然$(GlcNAc)_5$之純度只有 86%，但其餘的糖均在 92%以上。所以本研究以各純化後的糖為主要碳源，瞭解 9 種腸內菌利用的情形，是可信的。

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Table 1

Concentrations of each *N*-acetylchitooligosaccharides after assay of HPLC for fractionated peaks using Biogel P4

Fraction (Peak)	Concentrations of *N*-acetylchitooligosaccharides (mg/100 mL)						Yield(%)[a]
	GlcNAc	$(GlcNAc)_2$	$(GlcNAc)_3$	$(GlcNAc)_4$	$(GlcNAc)_5$	$(GlcNAc)_6$	
A	124.23(100)[b]	—[c]	—	—	—	—	12.6
B	1.56(1.3)	120.38(98.7)	—	—	—	—	8.2
C	0.57(0.4)	2.50(1.9)	130.25(96.8)	1.20(0.9)	—	—	7.9
D	0.73(0.4)	1.22(0.7)	4.50(2.6)	162.33(92.4)	6.23(3.5)	0.67(0.4)	11.7
E	0.07(0.1)	0.53(0.3)	2.53(1.4)	6.72(3.6)	159.98(85.9)	16.52(8.9)	7.1
F	0.50(0.2)	1.10(0.4)	3.50(1.3)	0.43(0.2)	5.8(2.2)	251.93(95.7)	3.8

[a] $\text{Yield}\ (\%) = \frac{\text{Dry weight of fractions collected (mg) for a peak}}{\text{Dry weight of total sample loaded (mg) into the column}} \times 100\%$

[b] The number inside parenthesis indicates percentage.

[c] Not detected

Table 2.

Maximum cell densities (MCD, log CFU ml^{-1}) and specific growth rates (μ, h^{-1}) of nine intestinal bacteria when cultivated in basal medium containing 0.2% (w/v) each individual $(GlcNAc)_{1\text{-}6}$ (1-6), or glucose (G) at 37℃ for 48 or 72 h under anaerobic condition

Bacterial strain and determination	1	2	3	4	5	6	G	B[a]
Bacteroides fragilis								
MCD, (4.33)[b]	9.20	8.89	8.92	8.86	8.69	8.57	8.98	8.66
μ	*0.525*	*0.157*	*0.164*	*0.165*	*0.181*	*0.205*	*0.571*	*0.096*
Bifidobacterium adolescentis								
MCD, (4.53)	7.32	7.31	7.26	7.24	7.23	7.23	9.82	7.20
μ	*0.050*	*0.034*	*0.034*	*0.034*	*0.034*	*0.034*	*0.877*	*0.034*
Clostridium perfringens								
MCD, (4.23)	7.54	8.80	8.75	8.70	6.59	6.56	8.77	6.53
μ	*1.172*	*1.619*	*0.854*	*0.834*	*0.834*	*0.824*	*1.410*	*0.814*
Escherichia coli								
MCD, (4.17)	8.13	8.08	8.10	8.08	8.06	8.04	8.30	7.94
μ	*0.496*	*0.454*	*0.427*	*0.439*	*0.404*	*0.389*	*0.549*	*0.343*
Eubacterium limosum								
MCD, (4.18)	7.85	7.77	7.61	7.64	7.70	7.63	8.54	7.52
μ	*0.069*	*0.065*	*0.065*	*0.065*	*0.060*	*0.060*	*0.244*	*0.060*
Lactococcus lactis								
MCD, (4.33)	8.92	8.85	8.21	8.21	8.04	7.98	9.08	7.51
μ	*0.711*	*0.694*	*0.596*	*0.596*	*0.569*	*0.560*	*0.729*	*0.488*
Proteus vulgaris								
MCD, (4.98)	8.18	8.14	8.12	8.11	8.09	8.02	8.26	7.97
μ	*0.518*	*0.414*	*0.406*	*0.398*	*0.396*	*0.390*	*0.614*	*0.382*
Staphylococcus aureus								
MCD, (4.08)	8.48	8.05	7.93	7.99	7.87	7.51	8.65	7.50
μ	*0.338*	*0.330*	*0.329*	*0.334*	*0.324*	*0.293*	*0.334*	*0.301*
Streptococcus faecalis								
MCD, (4.64)	8.79	8.74	8.73	8.71	8.72	8.37	8.85	8.53
μ	*0.678*	*0.673*	*0.663*	*0.648*	*0.637*	*0.635*	*0.763*	*0.613*

[a] Basal medium without adding sugar.

[b] Number in parenthesis indicates initial cell density (log CFU ml^{-1}).