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Chitooligosaccharides in Korean Commercial Salt-Fermented Shrimps, Determined by Enzyme-Linked Immunosorbent Assay

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Abstract In this study, we determined the content of chitooligosaccharides (COS) in Korean commercial salt-fermented shrimps by competitive direct enzyme-linked immunosorbent assays (cdELISAs), using anti-COS mixture (COSM) antibody and COSM horseradish peroxidase (HRP) conjugate. When COS6 was spiked into salt-fermented shrimps at the level of 10–300 μg/g, the average recovery was 120±19% (mean± S.D.). The COS contents of the 92 samples of Korean commercial salt-fermented shrimps collected during February 2000 and August 2002 were 36.3±20.7 μg COS6 equivalent/g (expressed as "μg/g" hereafter). Among the samples, the COS contents of *yuk-jeot* (40.3±22.5 μg/g, n=27) and *buksaewoo-jeot* (40.2±21.6 μg/g, n=5) were higher than the others. The COS contents of salt-fermented shrimps produced at Gwangcheon (47.1±20.7 μg/g, n=18) and Gomso (44.1± 21.8 μg/g, n=6) areas were higher than those produced at the other areas. This is the first report to determine COS of salt-fermented shrimps by cdELISA.

Key words: Salt-fermented shrimps, chitooligosaccharides, ELISA

In Korea, salt-fermented seafood (*jeot-kal*) has traditionally been favored and consumed as seasonings or further processed for fish sauce. Three major raw materials of *jeot-kal* among more than 150 kinds which are currently available in the market are anchovies, Alaskan pollack eggs, and small shrimps [10, 13]. Especially, salt-fermented shrimps (*saewoo-jeot*) have been used as a side dish for steamed rice and as seasonings for Kimchi in order to provide distinctive flavor [11]. They are produced by the action of endogenous hydrolyzing enzymes and/or natural microorganisms [21]. In 2002, the annual production of salt-fermented shrimps was 6,800 tons [1]. Salt-fermented shrimps (about 20,000 won/kg) are more expensive than the other *jeot-kal* (about 8,000 won/kg). Salt-fermented shrimps are classified mainly into *o-jeot*, *yuk-jeot*, *seha-jeot*, *buksaewoo-jeot*, *chu-jeot*, and *dongbaekha-jeot*, depending on the season of catch. Recently, many investigators have reported that *jeot-kal* possesses various functions such as antihypertensive [14, 18], fibrinolytic [3], and antioxidant effects [22]. However, there have been limited studies on the function of salt-fermented shrimps.

Numerous reports have indicated that chitin, chitosan, chitooligosaccharides (COS), and *N*-acetylchitooligosaccharides (NACOS) have various functions, such as anti-tumor [4, 7, 12], cholesterol lowering [16, 17], antibacterial [7], antifungal [5, 19], liver detoxifying [15], and anti-parasitic effects [6]. Park *et al.* [20] observed chitin oligosaccharides with molecular weight of 1 kDa from salt-fermented freshwater shrimps (*toha-jeot*) by gel permeation chromatography, although they did not present any direct evidences for COS.

An enzyme-linked immunosorbent assay (ELISA), has also been applied for the quantification of COS in the complex matrices of food. Kim *et al.* [9] produced the polyclonal antibody (Ab) specific to COS mixture (COSM) and developed competitive direct ELISA (cdELISA) for COS, and Eum *et al.* [2] and Kim *et al.* [8] applied the cdELISA to determine COS in *doenjang* (soybean paste) and *kanjang* (soy sauce). In addition, Shim *et al.* [23] developed cdELISAs for COS and NACOS using the anti-chitohexaose (COS6) and anti-*N*-acetylchitohexaose (NACOS6) Abs, respectively. In the present study, cdELISA was optimized and applied to determine COS and/or NACOS in Korean commercial salt-fermented shrimps.

cdELISAs were performed as follows; Ninety two commercial salt-fermented shrimps samples were collected from retail markets in the area of *Ganghwa*, *Gwangchun*, *Gomso*, *Jido*, and *Mokpo* during February 2002 and August 2002. Three g of the sample were added to 27 ml

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Table 1. Comparison of COS and NACOS contents in salt-fermented shrimps as determined by the cdELISAs using three antibodies.

cdELISA		Range	Mean±S.D. (n)[1]
Antibody	Unit		
(A) Anti-COS6	COS6 equiv. (g/g)[2]	2.7- 19.5	9.6±5.3 (12)
(B) Anti-COSM	COS6 equiv. (g/g)[3]	9.0- 35.0	18.8±7.6 (12)
(C) Anti-NACOS6	NACOS6 equiv. (g/g)[4]	0.1- 3.7	1.7±1.5 (12)

(A) For cdELISA, each well of the plates was coated with 100 µl of protein A/G (2 µg/ml) in the coating buffer overnight at 4°C. After washing three times, each well was treated with 100 µl of the anti-COS6 Ab (antiserum diluted 1/5,000 in the washing buffer (PBST)) for 1 h at R.T. After washing three times, each well was treated with 100 µl of 1:1 mixture (COS6 or samples:COS6-HRP conjugate diluted 1/15,000 in the washing buffer) and left for 1 h at R.T. Then, the substrate solution (H_2O_2/TMB) was added to each washed well, developed for 30 min, and finally the absorbance at 450 nm was measured. The concentration of COS6 extracted with PBST was determined in reference to the standard curve within a linear range.

(B) A cdELISA was performed according to the same procedure as described in the cdELISA (A), except the anti-COSM-BSA Ab (1/5,000) and COSM-HRP conjugates (1/10,000) were used instead of the anti-COS6 Ab and COS6-HRP conjugate, respectively.

(C) A cdELISA was performed according to the same procedures in the cdELISA (A), except the anti-NACOS6-BSA Ab (1/5,000), NACOS6, and NACOS6-HRP conjugates (1/3,000) were used instead of the anti-COS6 Ab, COS6, and COS6-HRP conjugates, respectively.

[1]Twelve samples of *yuk-jeot* (*Acetes japonicus*) were randomly selected and their COS or NACOS contents were determined by the ELISAs.

[2]The COS contents were determined with reference to the standard curve of COS6 in the cdELISA described in (A).

[3]The COS contents were determined with reference to the standard curve of COS6 in the cdELISA described in (B).

[4]The NACOS contents were determined with reference to the standard curve of NACOS6 in the cdELISA described in (C).

of phosphate buffered saline with Tween 20 (PBST: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl, and 0.05% Tween 20), the mixture was homogenized at 11,000 rpm for 2 min using an homogenizer (ULTRA TURRAX T25, JANKEL & KUNKEL, Germany). One ml of the extract was centrifuged at 9,500 ×*g* for 10 min, and the supernatant was then diluted about 1/100 in PBST. For cdELISA, each well of 96-well microplateMaxisorp™ (#446612), Nunc Co., Roskilde Denmark was coated with 100 µl of protein A/G (2 µg/ml) in the coating buffer (tris(hydroxymethyl)aminomethane, 0.05 M, pH 9.0) overnight at 4°C. After washing three times, each well was treated with 100 µl of the anti-COSM Ab (antiserum diluted 1/5,000 in the washing buffer) for 1 h at room temperature (R.T.). After washing three wells, 100 µl of 1:1 mixture of COS6 standard or sample and COSM-HRP conjugate were added into each well and left for 1 h at R.T. Then, to each washed well, 100 µl of a substrate solution (0.01% TMB, 0.05 M phosphate citrate buffer, pH 5, 0.002% H_2O_2) were added, developed at R.T. for 30 min, and the reaction was stopped with 50 µl of 2 M H_2SO_4. Absorbance at 450 nm was measured with a microplate reader (THERMOmax™ Molecular Devices Co., Sunnyvale, CA, U.S.A.). The ELISA procedures using the anti-COS6 and anti-NACOS6 Abs for COS and NACOS, respectively, were basically the same as for that using the anti-COSM Ab. COS and NACOS concentrations were expressed as COS6 and NACOS6 equivalents, respectively, which were determined with reference to the standard curve of COS6 or NACOS6.

In the previous study [23], specific antibodies (Ab) against three oligosaccharides (COS6, NACOS6, and COSM) were produced to develop ELISAs for the analyses of COS and/or NACOS. As a preliminary study, the cdELISAs were applied to determine COS and/or NACOS in 12 commercial salt-fermented shrimp samples. As listed in Table 1, the COS contents by the cdELISA using the anti-COSM and anti-COS6 Abs were 18.8±7.6 and 9.6±5.3 µg COS6 equivalent/g, respectively, however, the NACOS contents determined by the cdELISA using the anti-NACOS6 Ab were 1.7±1.5 µg/g NACOS6 equivalent/g. The COS contents were 5- 10 times higher than the NACOS contents, showing that not NACOS but COS are the main components of chitin or chitosan digests in salt-fermented shrimps.

The COS contents determined by the cdELISA with the anti-COSM Ab seemed to be about twice higher than those by the cdELISA with the anti-COS6 Ab. These results could be explained as follows; The cross-reactivities of the anti-COS6 Ab toward COS6 and COSM were 100% and 56%, respectively, and those of the anti-COSM Ab towards COS6 and COSM were 100% and 100%, respectively [23]. In the cdELISA using COS6 as a standard, the relative reactivity of the anti-COSM Ab toward COSM seemed to be higher than that of the anti-COS6 Ab toward COSM.

Therefore, it was suggested that digests of chitin or chitosan in salt-fermented shrimps might be similar to COSM, which are not completely deacetylated (97%). On the other hand, COS6 or NACOS6 is completely deacetylated or completely acetylated, respectively [23]. The COS contents of salt-fermented shrimps when determined by the cdELISA using the anti-COSM Ab and COS6 as a standard were almost the same as determined by the cdELISA using the anti-COS6 Ab and COSM as a standard (data not shown). COS6 is reagent pure and consists of a single component, but COSM is a mixture of digested chitosan with variable composition. Therefore, in this study, we used COS6 as a standard for the quantitation of COS in salt-fermented shrimps in the cdELISA using the anti-COSM Ab.

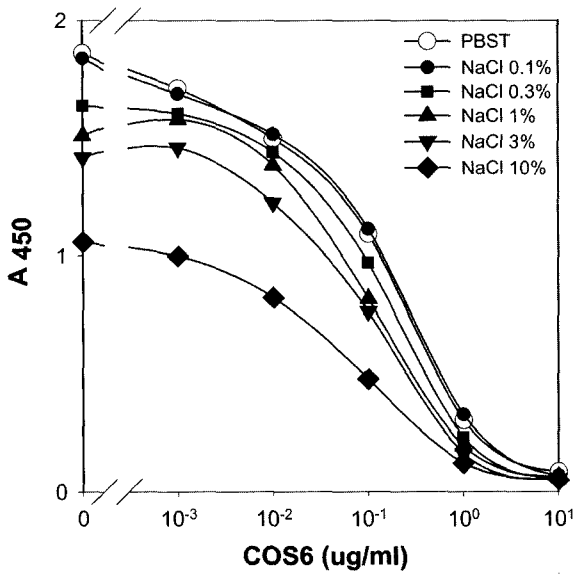

Fig. 1. Effect of salt on standard curves of cdELISA for detection of COS.
A 1:1 mixture (COS6:COSM-HRP conjugate diluted 1/1,000 in the PBST) was added to the anti-COSM-BSA antibody-coated plate. After 1 h, the plate was washed, the substrate solution (H_2O_2/TMB) was added, developed for 30 min, and finally absorbance at 450 nm was measured. NaCl at 0.1-10% final concentration was added to the PBST buffer.

Salt-fermented shrimps contained high salt concentration (about 20-25%, w/v), which might hinder the COS analysis by the cdELISA. Thus, the effect of salt on the cdELISA was examined. As shown in Fig. 1, when 10% (w/v) of NaCl were added to PBST, the cdELISA signals were lowered to half compared with control. However, addition of 0.1% NaCl indicated a standard curve profile almost similar to that of PBST only. Therefore, in this study, we diluted the salt-fermented shrimps extracts about 200 times or more with PBST.

Table 2. Recovery of COS6 spiked into salt-fermented shrimps[1] as determined by the cdELISA[2].

COS6 added (g/g)	COS6 equiv. detected[3] (g/g)	Recovery (%)
10	10±2.3 (23)	99.7
30	30±3.0 (10)	102
100	140±27 (19)	140
300	416±49 (12)	139
Overall		120
Recovery, %		19
S.D., %		16
C.V., %		

[1]Samples used were *yuk-jeot*.
[2]A cdELISA was performed according to the procedure described in Table 1(B).
[3]Mean of interassay (n=3)±S.D. (C.V., %). (COS6 detected after spike) - (COS6 detected before spike: 7 μg COS6 equivalent/g).

Table 3. COS contents of various Korean commercial salt-fermented shrimps[1] as determined by the cdELISA[2].

Sample	Range COS6 equiv. (g/g)	Mean S.D. (n) COS6 equiv. (g/g)
O-jeot	12.5-69.0	34.1±18.8 (13)
Yuk-jeot	11.3-90.0	40.3±22.5 (27)
Buksaewoo-jeot	19.4-74.0	40.2±21.6 (5)
Seha-jeot	1.5-52.0	26.4±19.5 (5)
Chu-jeot	4.2-90.0	39.7±19.5 (29)
Others[3]	6.0-42.2	22.6±12.7 (5)
Imported[4]	8.2-64.0	26.5±16.7 (8)
Overall	1.5-90.0	36.3±20.7 (92)[1]

[1]Ninety-two commercial salt-fermented shrimp samples were collected from retail markets during February 2000 and August 2002.
[2]A cdELISA was performed according to the procedure described in Table 1(B).
[3]Others were *detdegi-jeot* (n=2), *gogaemi-jeot* (n=1), *mix-jeot* (n=1), and *gonjaengi-jeot* (n=1).
[4]Imported samples were from China (n=5) and Vietnam (n=3).

The efficacy of the cdELISA for analysis of COS in commercial salt-fermented shrimps was assessed by recovery tests. Thus, COS6 in triplicates were spiked to 3 g of *yuk-jeot* at final concentrations of 10, 30, 100, and 300 g/g. After kept overnight at 4°C, COS contents of the spiked samples were analyzed by the cdELISA. The standard material for spike test was deduced to be 7 μg COS6 equivalent/g. The recovery of COS6 was 120±19% (mean S.D.) in the range of 10-300 μg/g (Table 2). Consequently, the cdELISA appeared to be possible to determine the COS in commercial salt-fermented shrimps in this range.

When analyzed by the cdELISA, the COS contents in 92 Korean commercial salt-fermented shrimps were 36.3±20.7 μg COS6 equivalent/g (expressed below as "μg/g") (Table 3). The COS contents of *yuk-jeot* (40.3±22.5 μg/g, n=27), *buksaewoo-jeot* (40.2±21.6 μg/g, n=5), and *chu-jeot* (39.7±19.5 μg/g, n=29) were higher than the average contents of all the above commercial salt-fermented shrimps. In particular, the COS contents of *yuk-jeot*, which is known to be the best quality and the most expensive among Korean commercial salt-fermented shrimps, were the highest in the samples tested. The COS contents of salt-fermented shrimps produced at the Gwangcheon (47.1±20.7 μg/g, n=18) and Gomso (44.1±21.8 μg/g, n=6) areas were higher than those of the others (32.1±19.0 μg/g, n=68). The COS contents of imported samples (26.5±16.7 μg/g, n=8) were lower than domestic ones (37.2±20.9 μg/g, n=84). However, the difference of COS contents among salt-fermented shrimps might have been due to small shrimps, fishing season, salt concentration, fermentation temperature, and fermentation period. More studies should be carried out to elucidate the effects of these parameters on the COS contents of salt-fermented shrimps.

In conclusion, this is the first study to determine the COS contents of commercial salt-fermented shrimps by cdELISA using the specific Ab.

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