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Detection of Chitooligosaccharides in Korean Soybean Paste by Tandem Immunoaffinity-ELISA

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Abstract In order to detect chitooligosaccharides (COS) in soybean paste, tandem immunoaffinity chromatography and enzyme-linked immunosorbent assay (ELISA) were developed. Polyclonal anti-chitooligosaccharides mixture (COSM) antibody specific to COSM was attached to Sepharose gel for initial sample cleanup and concentration of COS in soybean paste. COS was eluted and quantified by competitive direct ELISA (cdELISA). Average ELISA recoveries from the column using binding buffer spiked with COSM at levels of 0.5, 2.0, 5.0, and 10.0 µg/ml were 79.8, 72.0, 77.7, and 60.6%, respectively, with a mean recovery of 72.5%. Mean inter-well and inter-assay coefficients of variation (CV) were 7.7% and 10.3%, respectively. Average recoveries from soybean paste spiked with COSM at levels of 2, 6, 20, and 60 µg/g were 115, 91.7, 91, and 73.3%, respectively, with a mean recovery of 92.8%. Mean inter-well and inter-assay CV were 12.9% and 16%, respectively. The COS was detected from 24 out of 25 homemade Korean soybean paste samples at an average of 14.0 µg/g (n, 25; range, 0–51.2 µg/g) and from 13 out of 14 commercially made soybean paste samples at an average of 4.1 µg/g (n, 14; range, 0–18.4 µg/g). The tandem immunoaffinity chromatography-cdELISA that was developed in this study showed that the level of COS eluted from homemade soybean paste was higher than that of the commercially made ones. In addition, the level of COS eluted from commercially available soybean paste in Korea was higher than that of the ones in Japan.

Key words: Chitooligosaccharides (COS), immunoaffinity, ELISA, soybean paste

Chitosan, known as a cationic polymer of β-1,4-D-glucosamine, is rarely found in nature, but commercially available. It was derived by the chemical deacetylation of chitin from the shells of crab, shrimp, lobster, and krill etc. The primary occurrence of chitosan is as a cell wall component of *Mucor* [11], *Rhizopus* [13], *Absidia* [12], and *Saccharomyces* [2] in which they are found in several Korean fermented foods such as soybean paste (*Deonjang*), soy sauce, salted and fermented shrimp, and Korean turbid rice wine (*Takju*). There is a high probability of existing chitooligosaccharides (COS), especially in fermented food such as homemade *Mejoo* in Korea, because many microflora from environments are colonized in homemade *Mejoo*. During the fermentation process, these fungal chitosan in food can be hydrolyzed into COS, which has physiological functions such as immunopotentiating [6, 14], antitumor [7, 8, 16, 18], and antimicrobial activities [17]. Due to these physiological functions, COS can actually be used as food additives.

Conventional methods such as TLC [20], HPLC [19], and colorimetric method [15] have been used to detect the COS in multicomponent mixtures in certain foods, but those methods were not satisfactory. For example, the detection limit of COS is too high in HPLC analysis due to the low sensitivity level of the refractive detector. For fast and easy detection of COS, anti-chitooligosaccharides mixture (COSM) of polyclonal antibody specific to COSM was used, and competitive direct enzyme-linked immunosorbent assay (cdELISA) was established [9]. However, it was very difficult to detect the COS in complex matrix in certain foods which contain a relatively low amount of COS because the food samples should be diluted several times to eliminate the matrix effects on the cdELISA. In addition, the level of the COS in food was not high enough to be directly detected by cdELISA, although the cdELISA had a lower detection limit of COS than HPLC. Thus, it is necessary to remove the matrix effect of foods on the cdELISA and to concentrate a small of COS by using the immunoaffinity chromatography, although an immunoaffinity

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chromatography is used as a cleanup and a concentration step for making an instrumental analysis more feasible than ELISA [5]. Also, a more rapid and simple method is needed to detect low level of COS in foods better than HPLC.

In this study, an affinity chromatography was introduced as a cleanup and a concentration step of COS for cdELISA. Also, a tandem immunoaffinity chromatography and cdELISA for fast and easy detection of COS were developed and applied to the food samples, mostly in soybean paste.

MATERIALS AND METHODS

Materials

All inorganic and organic chemicals were reagent grade or better. Hydrogen peroxide (H_2O_2) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Chitooligosaccharides mixture (COSM) was prepared as described previously [9]. Briefly, chitosan was hydrolyzed with chitosanase I that was purified in the previous study [10] and the hydrolyzate was further purified with Bio-Gel p-4 column (1.2×90 cm). The positive fractions were pooled, lyophilized, and used as COSM. COSM-peroxidase conjugate (COSM-HRP) was prepared according to the manufacturer's instructions (ImmunoPure *Plus* Activated peroxidase Kit #31489, Pierce Co., Rockford, IL, U.S.A.) and the conjugate was purified with Sephadex G-25 column. Protein A column (ImmunoPure plus IgG Purification Kit #44697, Pierce Co.), and exocellulose column GF-5 (#1851850) were used for purification of anti-COSM polyclonal antibody, and disposable mini-columns were purchased from Pierce Co.

Immunoaffinity Gel

Purified anti-COSM antibody, which was produced in the previous study [9], was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B gel (Pharmacia Biotech, Uppsala, Sweden) as a method that was described in Azcona *et al.* [1]. Briefly, 1.25 g of S-4B was swollen and washed with 100 ml of distilled water. The gel was washed with 20 ml of coupling buffer (CB; 0.1 M of $NaHCO_3$, pH 8.3, 0.5 M of NaCl, Pierce Co., Rockford, IL, U.S.A.). Twenty mg of purified anti-COSM antibody was dissolved in 5 ml of CB. The gel suspension and the antibody solution were mixed end-over-end for 2 h at room temperature. After filtering, the remaining active groups on the CNBr-activated Sepharose 4B gel were blocked by placing the gel for 2 h at room temperature in 30 ml of glycine buffer (0.2 M, pH 8.0, Pierce Co.). After filtering, the excess blocking reagent and unattached antibody were discharged by washing the gel with four alternating cycles of high (CB) and low pH buffer (0.1 M of sodium acetate buffer, pH 4.0). Finally, the immunogel was washed with 0.01 M of phosphate buffered saline (PBS, pH 7.4), and it was stored at 4°C. The antibody concentration was measured before and after the coupling reaction by UV spectrophotometer and the degree of coupling was determined. The coupling yield was approximately 91.2%. According to the preliminary study, a buffer that should be used in immunoaffinity chromatography for the binding of COSM to the immunogel was tested with 0.1 M of PBS, pH 7.5 and the binding buffer (pH 8.0, Pierce Co. #21007) that was used in protein A column. As a standard deviation (SD) of the recovery by using PBS binding buffer was ten times higher than that of the binding buffer from Pierce Co., the following experiments were completed with the binding buffer from Pierce Co.

Competitive Direct ELISA (cdELISA)

Wells of the plate were coated with 100 µl of COSM-BSA conjugate (2 µg/ml) in a coating buffer (0.02 M of Tris, 0.15 M of NaCl, pH 9.0) as previously described [9]. Coated plates were washed three times with 300 µl of 0.02% of Tween 20 in washing buffer (0.02 M of Tris, 0.15 M of NaCl, pH 7.4). A 50 µl of samples or COSM standard along with 50 µl of COSM-HRP conjugate (diluted to 1:70 in a washing buffer) were added into coated wells. Plates were incubated for 1 h at 25°C, washed three times with washing buffer, and the coloring reaction with TMB was completed according to the same procedure as previously described [9]. All COS concentration from cdELISA were expressed as COSM equivalent, which was calculated from the standard curve by using a COSM as a standard.

Tandem Immunoaffinity Chromatography-cdELISA

Immunoaffinity columns consisted of 2 ml disposable columns containing 200 µl of immunogel slurry. Columns were equilibrated with binding buffer (pH 8.0, Pierce Co.) before the sample loading and the immunoaffinity chromatography studies were carried out at room temperature. The capacity of the immunoaffinity column was determined by passing an aliquot of 1 ml binding buffer containing COSM (0.5–10 µg) and cdELISA. Samples were applied onto the immunoaffinity column under atmospheric pressure with a rate of 0.2 ml/min. The column was washed with 5 ml of binding buffer and the bound COSM was eluted with 2 ml of elution buffer (0.1 M of glycine, 0.15 M of NaCl, pH 2.8). The level of COSM from this fraction was directly quantified by cdELISA. Columns were washed with additional 3 ml of elution buffer and re-equilibrated with 15 ml of the binding buffer for reuse.

Preparation of Soybean Paste Sample

Twenty-five samples collected from all over the country consisted of homemade soybean pastes, which were made

according to the traditional method. Fourteen samples of commercially available soybean pastes were purchased from retail stores. At first, the collected samples were freeze-dried and pulverized. Twenty ml of distilled water was added into 2 grams of each pulverized sample and heated at 120°C for 15 min to extract COS. The boiled samples were filtered through a Whatman No. 1 filter paper and the filtrates were centrifuged at 10,000 ×g for 20 min. Three ml of the supernatant and 3 ml of the binding buffer were mixed and applied onto the immunoaffinity column. Then, the column was washed with 5 ml of binding buffer and the bound COS was eluted with 2 ml of elution buffer. Each eluent was assayed by cdELISA without any dilution. The spiked samples were prepared by adding COSM stock solution to binding buffer to give final concentrations of 0.5, 2.0, 5.0, and 10.0 μg/ml, respectively, and to the soybean paste extracts to give a final concentrations of 2, 6, 20, and 60 μg/g, respectively.

RESULTS

Characteristics of Immunoaffinity Column

As the theoretical maximum capacity of affinity column was 16 μg/column on the base of chitohexaose, the spiking level of COSM was completed within this range. The recovery of COSM from the affinity columns was tested and the results are shown in Table 1. When COSM was spiked with the binding buffer, the detected level of COSM in 10 μg/ml was a little lower than those of others. The overall recovery was 72.5% with 10.3% of the coefficient variation (CV). The recovery test showed that the column can be stably used in a range of 0.5- 10 μg/ml COSM.

Table 1. Recovery of COSM dissolved in a binding buffer as determined by tandem immunoaffinity chromatography-cdELISA.[1]

COSM added (μg/ml)	COSM detected[2] (μg/ml)	Recovery (%)
0.5	0.40±0.01 (1.0)	79.8
2.0	1.44±0.18 (12.5)	72.0
5.0	3.88±0.35 (9.1)	77.7
10.0	6.06±0.41 (6.8)	60.6
Mean of CV, %	(7.7)	
Overall recovery, %		72.5
SD, %		3.9
CV, %		10.3

[1]One ml of individual concentration of COSM was applied onto the immunoaffinity column, washed, and eluted. Fifty μl of each eluent was directly used for cdELISA. CdELISA was completed as follows; A 1:1 mixture (eluent: COSM-HRP conjugate diluted to 1/70 in the washing buffer) was added to the anti-COSM antibody-coated plate, and the plate was incubated for 1 h. After waching, the substrate solution (H_2O_2/TMB) was added, developed for 30 min, and finally the absorbance level was measured.

[2]Mean of inter-well (n=3) ±SD.

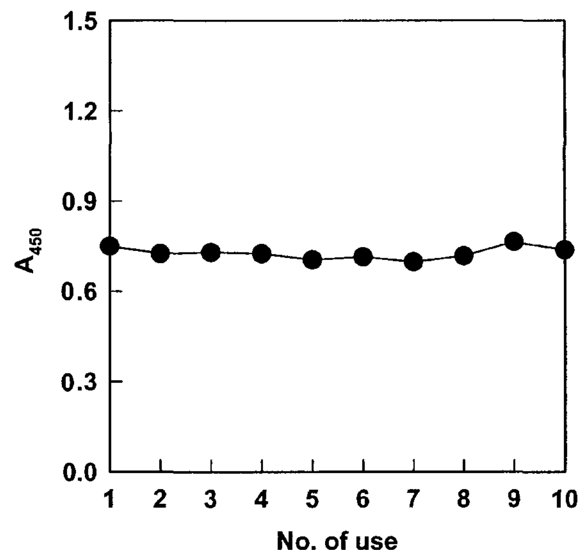

Fig. 1. Regeneration degree of immunoaffinity column.
Each time 1 ml of COSM (1 μg/ml) was loaded onto the immunoaffinity column. The column was washed with 5 ml of binding buffer (pH 8.0, Pierce Co., Cat #21007) and COSM was eluted with 2 ml of elution buffer (0.1 M of glycine, 0.15 M of NaCl, pH 2.8). The eluent was used for cdELISA. Column was washed with additional 3 ml of elution buffer and re-equilibrated with 15 ml of binding buffer. CdELISA was previously described in Materials and Methods.

The affinity columns (0.2 ml volume size) are reusable several times after eluting bound COSM with elution buffer and re-equilibrating with binding buffer. As shown in Fig. 1, 10 samples were passed through the immunoaffinity column

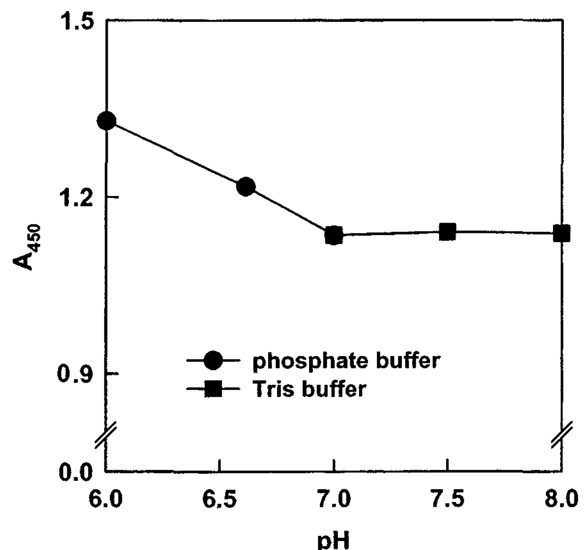

Fig. 2. Effect of competition buffer pH on antigen-antibody interaction in cdELISA.
Hundred μl of 1:1 mixture (COSM, 0.5 μg/ml in each indicated pH buffer: COSM-HRP conjugate diluted to 1/70 in the washing buffer) was used for cdELISA. CdELISA was previously described in Materials and Methods.

without any loss of binding capacity. The affinity resin made of CNBr-activated Sepharose 4B gel has a tendency to lose antibody activity by acidic elution buffer, but this affinity column can be reusable more than 10 times.

When detecting COS by cdELISA, the eluent pH from an immunoaffinity column should be above 7. 0. As shown in Fig. 2, the OD was influenced by the pH of competition buffer in the cdELISA, especially below pH 7.0. Thus, the eluent of immunoaffinity column was tested at low pH of 2.8. However, the results showed that low pH did not affect the pH of elution from the immunoaffinity column, since the binding buffer used in this procedure could neutralize 2 ml of the eluent. Therefore, it was not necessary to adjust pH after elution.

Detection of the COS in Soybean Pastes

To evaluate the accuracy of the tandem immunoaffinity chromatography-cdELISA, the COS in soybean paste, and the COSM were spiked with soybean paste extracts and the recoveries were determined. In the recovery test, ideally the soybean paste extracts should not contain any COS. The soybean paste made in Japan, in which COS was not detected by applying the tandem immunoaffinity chromatography-cdELISA, was selected in order to correct the effect of soybean paste extract on the cdELISA. According to the experiment of Azcona *et al.* [1], the recovery values of zearalenone in water and milk were 95%and 126%, respectively so these values were quite different. Thus, COSM was spiked with the Japanese soybean paste, in which COS was not detected, and this was done in order to obtain a reliable recovery of COSM from the soybean paste. As shown in Table 2, 2-60 μg/g of recovery of COSM was so broad that the SD and CV were 14.8% and 16% respectively, and these values were higher than those in binding buffer. In addition, the recovery rate at a level of 2 μg/g and 60 μg/g spike was 116% and 73.3%, respectively, although the average recovery rate was 92.8%. The recovery test showed that the column can be stably used in a range of 0.5-60 μg/ml COSM.

Using this tandem immunoaffinity chromatography-cdELISA method, the detection of COS from soybean paste samples was performed. As shown in Table 3, the detection level of COS was in a range of 0-51.2 μg/g for 25 samples of homemade soybean paste and this detection level was classified into 3 groups such as less than 1 μg/g (1st group), 1-10 μg/g (2nd group), and greater than 10 μg/g (3rd group). Three, ten, twelve samples belonged to the first, second, and third group, respectively. In the case of 14 commercially available soybean pastes including two Japanese samples, three, nine, and twelve samples belonged to the first, second, and third group, respectively.

According to the overall results, it seems that homemade soybean paste samples contained higher amounts of COS

Table 2. Recovery of COSM spiked into Japanese soybean paste as determined by tandem immunoaffinity chromatography-cdELISA.[1]

COSM added (μg/g)	Detected[2] (μg/g)	Recovery (%)
2	2.3±0.4 (17.4)	116.0
6	5.5±0.3 (5.5)	92.3
20	18.2±3.5 (19.2)	91.0
60	44.0±4.1 (9.3)	73.3
Mean of CV, %	(12.9)	
Overall recovery, %		92.8
SD, %		14.8
CV, %		16.0

[1]The COSM spiked soybean paste sample was extracted with distilled water at 120°C for 15 min, filtrated, and centrifuged. The mixture of 3 ml of supernatant and 3 ml of binding buffer was applied onto the immunoaffinity column, washed, and eluted. The eluent was used for cdELISA in the same manner as shown in Table 1.

[2]Mean of inter-well (n=3) ±SD.

Table 3. Amount of COS as a COSM equivalent in soybean paste by tandem immunoaffinity chromatography-cdELISA.[1]

HM[2]		COM[3]	
Sample No.	COS (μg/g)	Sample No.	COS (μg/g)
H1	11.1	C1	4.3
H2	10.2	C2	18.4
H3	7.5	C3	2.1
H4	9.8	C4	1.5
H5	51.2	C5	1.4
H6	24.6	C6	1.0
H7	8.7	C7	13.4
H8	11.9	C8[5]	N.D.[4]
H9	23.4	C9	2.2
H10	1.5	C10	1.0
H11	11.0	C11	2.5
H12	2.8	C12[5]	1.1
H13	32.3	C13	1.3
H14	6.7	C14	7.0
H15	14.6		
H16	0.5		
H17	4.4		
H18	9.5		
H19	0.8		
H20	38.9		
H21	10.4		
H22	N.D.[4]		
H23	1.9		
H24	23.1		
H25	34.1		
No. of samples	25	No. of samples	14
Range (μg/g)	0-51.2	Range (μg/g)	0-18.4
Mean±SD (μg/g)	14.0±10.4	Mean±SD (μg/g)	4.1±3.8

[1]Samples were extracted in the same manner as shown in Table 2. CdELISA was performed in the same manner as shown in Table 1.

[2]Traditionally made Korean soybean paste.

[3]Commercially made soybean paste.

[4]N.D.: not detected.

[5]Japanese type soybean paste.

than commercial soybean paste. The level of COS of two commercial soybean pastes was as high as that of the homemade soybean pastes, and the COS level of 3 homemade soybean pastes was as low as that of the commercial soybean pastes. However, the COS was not detected in one Japanese sample and barely detected in the other one (1.1 μg/g). Among the commercially available soybean pastes, the COS level of Korean soybean pastes was much higher than that of Japanese one, even though the number of samples was small. According to the above result, on the average, the homemade soybean pastes contain higher levels of COS compared to commercially available soybean pastes, although the level of COS in some samples of homemade ones was hardly or not detected at all and high level of COS was actually detected in a few commercially available soybean pastes.

Discussion

A cdELISA was established with anti-COSM antibody in the previous study [9]. This cdELISA was used to detect the level of COS in soybean paste (*Deonjang*). The extracted spiked soybean paste samples were diluted in a series and used for the analysis, but the result was mostly influenced by dilution ratio of the extracted samples (data not shown). The reliable recovery could be obtained in a high fold dilution of the extract. However, the detection limit was increased. Thus, an immunoaffinity chromatography for the sample cleanup step was devised to detect the COS which slightly exists in food samples.

It seems that the homemade soybean paste samples contain higher amounts of COS than the commercially produced ones on the average. However, the level of COS in some samples of homemade ones was hardly or not detected at all, whereas high level of COS was detected in two samples of commercially produced soybean pastes.

Theoretically, it is more reasonable to classify that a fungal flora of soybean paste belongs to *Zygomycetes* such as *Rhizopus*, *Mucor*, and *Absidia* etc. or yeast with chitosan as a cell wall component rather than other fungal flora due to the production of COS in soybean paste. When considering most of commercially available soybean pastes made only with *Aspergillus oryzae Koji*, none or small amounts of COS would be expected in those samples. However, a low level of COS was detected in some of those samples (Table 3). A chitin, a cell wall component of *Aspergillus* sp., can be deacetylated to chitosan by some enzymes involved in cell wall hydrolysis and chitin deacetylase can be produced from bacteria during the *Mejoo* fermentation process. In a course of the enzyme catalyzed reaction, hetero-chitooligosaccharides (hetero-COS) and COS might be produced and detected by cd-ELISA. According to Grenier *et al.* [4], they studied the cell wall component of *Aspergillus niger* with chitosanase-gold complex by cytochemical labelling and explained that the cell wall, chitin, was partially deacetylated, and generally bound by chitosanase labelled with chitosan. This report could support the fact that the low amount of COS was detected in the commercial soybean paste samples made with *Aspergillus Koji*. On the other hand, higher level of COS detected in four samples of commercial soybean pastes may be caused by a contamination of *Zygomycetes* and/or some species of yeast with chitosan as a cell wall component during the *Mejoo* processing [2]. In contrast, the result shown with a small amount of the COS in some of the homemade soybean pastes (Table 3) could be considered as those which were not made with traditional *Mejoo*, but with purchased *Mejoo* having *Aspergillus Koji*.

The physiological functions of COS have been reported in the fields of medicine and food [6, 7, 8, 14, 17, 18]. From this point of view, fermented foods, like soybean paste, can be considered as a functional foods especially that contain *Rhizopus*, *Absidia*, *Mucor*, and Yeast as COS source. Therefore, the mix-cultured *Mejoo* made by following the traditional process offers high level of COS that produces a more physiologically functional soybean paste than the commercially available ones. Furthermore, tandem immunoaffinity-cdELISA could be applied to the detection of COS in other types of food samples, which may contain low level of COS during the fermentation process such as soy sauce, salt-fermented shrimps, and *Takju* (Korean turbid rice wine) etc.

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