A Modified Chemical Procedure for Rapid Determination of Glucosamine and Its Application for Estimation of Mold Growth in Peanut Kernels and Koji

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One-step hydrolysis of chitin to release glucosamine for quantitation was achieved by combining a chitin-containing sample (10−200 mg of sample size) in a test tube with 1 mL of 10 M HCl followed by vacuum treatment for 10 min, incubation at 28 °C for 30 min, replenishment with 3 mL of deionized water, nitrogen flushing, screw capping, and heat treatment at 140 °C for 60 min. A phosphate buffer solution (pH 12.5, 0.2 M) was effective in pH stabilization and enhancing colorimetric determination of glucosamine content. When the modified procedure was applied to analyze glucosamine content in the mycelia of various molds, glucosamine content varied mainly depending on mold species. In estimations of mold growth of the uninoculated peanut kernels incubated under a humidified condition for 5 weeks, cooked rice and soybean inoculated with conidia of *Aspergillus oryzae* for koji preparation, logarithms of the internal mold populations and glucosamine contents both increased with increases of incubation time. The modified procedure provided a rapid and reliable estimation of mold growth in various substrates.

Keywords: *Glucosamine; chitin; MBTH; buffer solution; internal mold population; peanut; koji*

INTRODUCTION

Mold infection and subsequent production of mycotoxin in foods is a matter of concern for public and food microbiologists. A reliable and accurate detection of mold infection is essential in defect sorting from raw material and ingredients destined for food use. In addition to methods used for direct enumeration of fungal propagules, chitin determination of fungal cell wall is often conducted as a means for assessing the extent of fungal infection of foods (Gourama and Bullerman, 1995; Cousin, 1996). Since chitin is not soluble in water and most solvents, hydrolysis with acid, alkali, or enzymes to yield glucosamine for chemical determination is necessary. Characteristics of release of glucosamine from chitin subjected to hydrolysis under various conditions have been extensively investigated (Ride and Drysdale, 1971, 1972; Bethlenfalvay et al., 1981; Holan et al., 1980; Plassard et al., 1982; Gurudiddaiah, 1978; Desgranges et al., 1991; Boyle and Kropp, 1992). All of these hydrolytic practices have indicated that acid hydrolysis is the preferred method (Cousin, 1996). In general, chitin can be hydrolyzed with acid to cleave the $\beta(1-4)$ glycosidic bonds, remove the acetyl group, and produce glucosamine for colorimetric quantitation. During acid hydrolysis, temperature, time, concentration of HCl, and headspace composition, that is, nitrogen flushing and screw sealing, are prime attributes affecting glucosamine recovery. An excess of acid treatment results in the breakdown of glucosamine and a decrease of recovery. Among those reported methods, most are laborious and time-consuming and glucosamine recovery varies by case.

Colorimetric reaction of glucosamine with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) and ferric chloride reported by Tsuji et al. (1969) has been demonstrated as a sensitive spectrophotometric method for glucosamine quantitation. Ride and Drysdale (1972) have further modified the method as a rapid procedure for chemical determination of filamentous fungi in plant tissue. Since then many research projects have been done using the modified method of Ride and Drysdale (1972) (Cousin, 1996). However, the effect of pH on glucosamine quantitation in the chitin hydrolysates has been meagerly investigated. Tsuji et al. (1969) have suggested that a mildly acidic condition is appropriate for color formation and quantitation. Desgranges et al. (1991) adjusted the pH of chitin hydrolysate to 7.0 with 10 and 0.5 M NaOH through a pH meter prior to color formation. However, the adjustment may result in an uneven final volume among samples and require volume manipulation or calibrations. A more accurate, reliable, and rapid procedure for chitin determination is still needed.

In this study, with an attempt to shorten the time of acid hydrolysis, a rapid one-step procedure was pursued and monitored mainly following the method of Desgranges et al. (1991) from which a two-step hydrolysis procedure lasting more than 16 h has been conducted. The modification included vacuum treatment, incubation of the chitin samples with HCl, temperature elevation, and increase of HCl concentration during thermal treatment for acid hydrolysis. The colorimetric reaction used was that of Ride and Drysdale (1972) with a minor modification. For the stabilization of the pH value of the chitin hydrolysates when subjected to serial dilution and colorimetric reaction, the pH value was adjusted and stabilized with a phosphate buffer solution. The modified procedure was further applied for the estimation of mold growth in peanut kernels incubated under a humidified condition for natural mold contamination. It was also tested respectively in cooked rice and

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soybean after inoculation with conidia of *Aspergillus oryzae* and incubation for koji preparation.

MATERIALS AND METHODS

Colorimetric Determination of Glucosamine As Affected by pH and Buffer Solution. A series of glucosamine (Sigma G-4875, Sigma Chemical Co., St. Louis, MN) solutions including 0.05, 0.1, 0.3, and 0.5 mg/mL were prepared. The pH values of the solutions were measured with a pH meter. From each solution, 2.5 mL was withdrawn and deposited in a series of beakers and each was replenished with 15 mL of deionized water. Then the pH values of the solutions were adjusted to 7.0 using 1.0 N NaOH (Desgranges et al., 1991) or 0.2 M, pH 12.5, sodium phosphate buffer solution. After pH adjustment, the solution was adjusted to 25 mL with deionized water. The pH values of the 25 mL solutions were measured again with a pH meter. Then, from each solution, 5 mL was withdrawn and mixed with 45 mL of deionized water for a 10-fold dilution. The diluted solutions were further subjected to pH measurement followed by colorimetric determination of glucosamine content.

The colorimetric reaction basically followed the method reported by Ride and Drysdale (1972) with minor modifications. Glucosamine-containing solution (350 μL), 325 μL of $NaHSO_4$ (5%, w/v), and 325 mL of $NaNO_2$ (5%, w/v) were added to each centrifuge tube (Nalgene 3114) and combined. Then the tubes were capped and incubated for deamination on a rocking plate (ca. 40 swings/min) for 15 min at room temperature. After addition of 1 mL of 12.5% (w/v) $NH_4SO_3NH_2$ each tube was mixed thoroughly on a vortex. The tubes were heated in a boiling water bath for 3 min and cooled to the ambient temperature with tap water followed by the addition of 0.5 mL of freshly prepared 0.5% (w/v) MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) solution. After the addition of 0.5 mL of $FeCl_3$ (0.5%, w/v) and incubation at 28 °C for 30 min, the solution was centrifuged (8500*g*, at 20 °C for 3 min) and absorbance at 650 nm of the supernatant was measured spectrophotometrically. As a blank, aliquots of 17.5 mL of deionized water were subjected to the same procedure for pH adjustment using 0.2 M, pH 12.5, sodium phosphate solution to pH 7.0 and colorimetric determination concurrently.

Chitin and Acid Hydrolysis. A crude crab chitin (Sigma C-9213, Sigma Chemical Co., St. Louis, MO) was used as a chitin source. Approximately 50 g of chitin was suspended in 200 mL of 5 M H_2SO_4, mildly agitated for 15 min using a magnetic stirrer, and held at room temperature for settlement. After the supernatant was discarded, the particulate pellet was washed three times with sufficient amounts of deionized water. The final material was collected by filtration through a filter paper and dried overnight at 60−65 °C. Then, it was pulverized with a cyclone coffee mill and designated as an acid-treated chitin and used for glucosamine quantitation in this study.

In the search for a one-step chitin hydrolysis method, we conducted extensive preliminary studies on sample treatment including vacuum treatment, nitrogen flushing, and 30 min incubation at 28 °C, HCl concentration, temperature, and duration for thermal hydrolysis. An optimized procedure was developed and is described in this report. To each test tube (10 mL) containing an acid-treated chitin sample (20 mg) was added 1 mL of 10 M HCl, and the mixture was vacuum-treated in a desiccator for 10 min, screw-capped, and incubated at 28 °C for 30 min. Then, 3 mL of deionized water was added, and the headspace was nitrogen-flushed and screw-capped for thermal hydrolysis with a thermal module (Pierce Co., Richmond, VA) at 140 °C for 60 min. The capped tubes were cooled with tap water to ambient temperature prior to neutralization of each hydrolysate with 1 mL of 10 M NaOH and replenishment with 1 mL of deionized water to comprise 6 mL of the final volume. From each tube, 2.5 mL was withdrawn and deposited in a 50 mL beaker; 15 mL of deionized water was added and followed by pH adjustment to 7.0 using 0.2 M, pH 12.5, sodium phosphate buffer solution. Then, each solution was transferred to a volumetric flask and replenished with deionized water to reach 25 mL of the final volume. A 350 μL aliquot of the solution was withdrawn and deposited into a centrifuge tube (Nalgene 3114). Then, the procedure described above for colorimetric determination of the glucosamine content was followed. The relationship between absorbance at 650 nm and glucosamine concentration obtained from the buffered glucosamine solutions was used as a working curve for the estimation of glucosamine content in the following experiments. As a control for comparison, the two-step hydrolysis procedure of Desgranges (1991) was followed with minor modifications. The test tube containing 20 mg of the acid-treated chitin sample and 1 mL of 10 M HCl was kept overnight (16 h) prior to the addition of 3 mL of deionized water. Next, the tube was nitrogen-flushed, screw-capped, and hydrolyzed at 140 °C for 60 min, and then the same procedure described above for glucosamine quantitation was followed.

Colorimetric Determination of Glucosamine in Acid Hydrolysate of Chitin as Affected by pH Adjustment. For comparison of pH effect on color intensity formed with MBTH, a series of beakers each containing 2.5 mL of the acid hydrolysate solution (withdrawn from the final 6 mL of acid-hydrolyzed solution) were mixed with 15 mL of deionized water. Then, each solution was subjected to pH adjustment with 0.2 M, pH 12.5, phosphate buffer solution or with 2.0 and 0.1 N of NaOH to pH 6.0, 7.0, and 8.0, respectively. Meanwhile, a series of phosphate buffer solutions (pH 12.5) with various concentrations including 0.1, 0.2, and 0.5 M were applied for comparison. After pH adjustment, additional deionized water was added to reach 25 mL, followed by a 10-fold dilution with additional deionized water and colorimetric determination of glucosamine content. As a blank, 17.5 mL of deionized water was subjected to pH adjustment to pH 7.0 with 0.2 M, pH 12.5, sodium phosphate buffer solution, and then the solution was adjusted to 25 mL with deionized water. Five milliliters of the latter was withdrawn and mixed with 45 mL of deionized water for a 10-fold dilution prior to colorimetric determination.

Mycelial Production of Various Molds and Chitin Determination. Various mold species including *Actinomucor tiwanensis, Aspergillus flavus, Aspergillus niger, A. oryzae, Penicillium citrinum, Penicillium digitatum,* and *Rhizopus microsporus* were used for mycelial production. From each slant culture, a loopful of conidia was inoculated into a 500 mL flask containing 200 mL of yeast-malt (YM) broth, followed by incubation at 28 °C in a shaker bath (120 rpm). After one week of incubation, the mycelial clumps were filtered through a four-layered cheesecloth enhanced by using a vacuum pump and washed several times with sufficient amounts of deionized water. The mycelial mass was dried at 60−65 °C for 24 h and ground with a mortar and pestle to prepare a mycelial powder to be used for glucosamine determination.

For determination of the glucosamine content in the mycelial powders, 10, 20 and 40 mg from each sample were weighed and deposited into a series of test tubes following the procedure of one-stop acid hydrolysis and colorimetric quantitation. The glucosamine content was estimated according to the standard working curve described above and expressed as a percentage on the dry weight basis.

Peanut Kernels and Mold Infection. Freshly harvested, sun dried, shelled, hand-sorted, and size-graded peanut kernels (Tainan 9, a Spanish cultivar) were used in this study. Kernels were sealed in polypropylene bags and stored at −25 °C until used. Sealed bags of kernels were removed from the freezer and tempered at ambient temperature (25−30 °C) overnight before opening. Twenty-five sound kernels were placed in each of several Perti dishes. The Petri dishes were placed on a rack above water in a sealed desiccator and incubated at 27 °C for assessment of the growth of the naturally contaminated molds. The position of dishes was changed every 3 or 4 days. One Petri dish containing peanuts was removed at 0, 1, 2, 3, and 5 weeks and placed in sealed polyethylene plastic bags and stored at −25 °C until further analyses.

Estimation of Internal Mold Population and Determination of Glucosamine Content of Peanut Kernels. Internal mold populations of the peanut kernels were deter-

mined following the methods of Pitt et al. (1993) and Chiou (1997) with minor modification. From each batch of peanut kernels subjected to various extents of mold infection, 5 kernels were deposited into a polyethylene plastic bag and weighed. Then the kernels were surface sterilized with 20 mL of 1.0% of NaOCl solution (prepared from a household Clorox solution containing 5.0% of NaOCl) for 1 min and drained, followed by disinfection with 20 mL of 75% ethanol solution for 30 s. After the ethanol solution was drained, the kernels in PE bags were coarsely cracked with a hammer and transferred into a dilution bottle containing 22.5 mL of sterile water, followed by homogenization with a polytron (Kinematica AG, Luzern, Switzerland) equipped with an aggregate (PT-DA 3012/2) operated at 15000 rpm for 1 min. After a serial dilution with diluent (0.1% peptone), 0.1 mL of the suspension was surface-spread onto AFPA (*Aspergillus flavus/parasiticus agar*) (Pitt et al., 1983). The medium contained 1.0% of bacteriological peptone, 2.0% of yeast extract, 0.05% of ferric ammonium citrate, 0.02% of chloramphenicol, 0.1% of a 0.2% dichloran stock solution in ethanol, and 1.5% of agar. The Petri dishes were incubated at 28 °C for 5 days for colony formation and enumeration of the internal mold population by counting the colony-forming units (CFU).

For chitin determination, the polytron homogenates were centrifuged (10000 rpm, 20 min at 20 °C, Hitachi SCR-20B) and the pellets were collected, placed on a filter paper, and dried at 60−65 °C. From each dried and pulverized sample, 200 mg of the powder was weighed, deposited into a test tube, and subjected to chitin determination following the above procedure. Peanut powder prepared from sound and mature kernels was used as a blank.

Estimation of Internal Mold Population and Determination of Glucosamine Content of Koji. Rice and soybean koji were prepared following the procedure of Chiou et al. (1997). When the cooked rice and soybean were cooled to below 40 °C, they were respectively inoculated with *A. oryzae* (inocula were ca. 7 log CFU/g) and incubated at 25−29 °C. During incubation, koji samples were taken at 0, 6, 21, 45, and 72 h and subjected to determinations of moisture content, internal mold population, and glucosamine content. For moisture content determination, each 3 g sample was mixed with 1 mL of 1.0% NaOCl solution for pasteurization due to hygiene consideration and heated in an oven at 105 °C until their constant weights were reached. For determinations of internal mold population and glucosamine content, 10 g koji samples were mixed with 25 mL of 1.0% NaOCl solution for 1 min and drained to discard the solution. The remaining koji was mixed with 40 mL of deionized water and homogenized with a polytron equipped with an aggregate (PT-DA 3012/2) at 15000 rpm for 1 min. Five milliliters of the homogenate was withdrawn and mixed with 95 mL of 0.1% peptone solution, and the mixture was further diluted with the diluent. After appropriate dilution, 0.1 mL aliquots were surface-spread onto AFPA medium for incubation. For determination of glucosamine content, the polytron homogenates were centrifuged (10000 rpm at 20 °C for 20 min, Hitachi SCR-20B) and the pellets were collected and oven-dried at 60−65 °C for 24 h. Then the dry powders were subjected to analysis of glucosamine following the above procedure.

Statistical Analysis. At least duplicate experiments were conducted. Means with standard deviation for the determinations of glucosamine content are reported. Linear regression and correlation efficiency (R^2) between specified variables were analyzed using JMP software (SAS Institute Inc.).

RESULTS AND DISCUSSION

When a series of glucosamine solutions with concentrations ranging from 0.05 to 0.5 mg/mL were prepared and subjected to pH measurement, the pH value of the solutions decreased from 6.9 to 5.8 with an increase in concentration (Figure 1). Since the role of pH is important in affecting chemical reactivity, pH adjustment is usually a necessary step in the control of a chemical reaction. Adjustment of the pH value with NaOH and HCl is the most common means practiced in laboratories. However, an appropriate dilution to adjust solute concentration for reaction is necessary to obtain a satisfactory result from a concentrated sample. Concentration adjustment by a serial dilution after pH adjustment may cause a further pH variation and volume change. When the 2.5 mL aliquots of glucosamine solutions with various concentrations were combined with 15 mL of deionized water and subjected to pH adjustment with NaOH or 0.2 M, pH 12.5, phosphate buffer, the pH value changed slightly in the NaOH-adjusted solutions after volume adjustment to 25 mL with deionized water (Figure 1). When the solutions were further subjected to a 10-fold dilution, a wider range of pH variation was observed in the NaOH-adjusted solutions. In comparison, the pH values of the buffer-adjusted solutions were resistant to change during volume adjustment to 25 mL and following 10-fold dilution with deionized water.

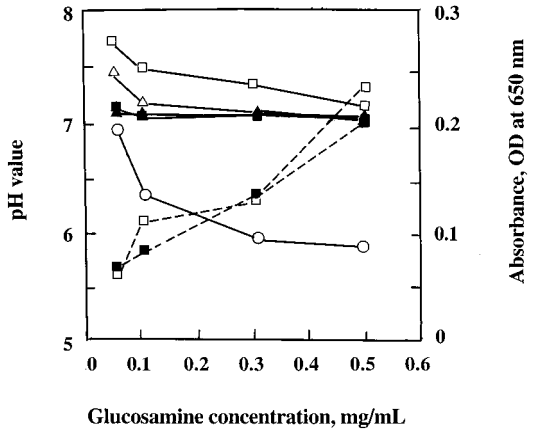

Figure 1. Changes in pH and absorbance of 2.5 mL aliquots of glucosamine solutions with concentrations ranging from 0.05 to 0.5 mg/mL while each was combined with 15 mL of deionized water and subjected to pH adjustment to 7.0 with a phosphate buffer solution (buffered) or with NaOH (nonbuffered) followed by volume adjustment to 25 mL and a further 10-fold dilution with deionized water for colorimetric quantitation of glucosamine: (—) pH; (---) absorbance at 650 nm; (○) original glucosamine solutions; (▲) buffered 25 mL solutions; (△) nonbuffered 25 mL solutions; (■) buffered and 10-fold diluted solutions; (□) nonbuffered and 10-fold diluted solutions.

When the finally diluted solutions were subjected to colorimetric reaction with MBTH and spectrophotometric measurement, absorbance at 650 nm of the samples increased with an increase of concentration (Figure 1). After linear regression, the correlation efficiencies (R^2) were 0.89 and 0.96 for the nonbuffered (NaOH-adjusted) and buffered glucosamine solutions, respectively. In comparison, phosphate buffer was effective in pH stabilization and resulted in a better linear relationship than that obtained from the nonbuffered solutions. The pH values of NaOH-adjusted and 10-fold diluted solutions were higher than 7.0 as affected by glucosamine concentration. For colorimetric quantitation of glucosamine content with MBTH, Tsuji et al. (1969) have suggested that a mildly acidic solution is appropriate. Desgranges et al. (1991) adjusted the pH of the chitin hydrolysate to 7.0 prior to color formation. Stabilization of pH at 7.0 seems essential for the achievement of a linear relationship as a working curve.

For constructing a working curve, the relationship between absorbance at 650 nm and glucosamine concentration obtained from the buffered glucosamine

Table 1. Glucosamine Contents and Relative Recoveries of Chitin Samples Subjected to Two-Step and One-Step Hydrolysis and Various Pretreatments Including Vacuum Treatments and Hydrolysis Times

acid hydrolysis of chitin and pH control for glucosamine quantitation	glucosamine content,[a] %	relative glucosamine recovery,[a] %
two-step acid hydrolysis[b]	45.1 ± 0.1	100
one-step acid hydrolysis		
without vacuum treatment	35.8 ± 0.7	79.4 ± 1.5
vacuum treatment and 30 min hydrolysis	44.2 ± 0.3	98.0 ± 0.7
vacuum treatment and 60 min hydrolysis	61.3 ± 0.7	135.9 ± 1.5
vacuum treatment and 90 min hydrolysis	63.7 ± 0.1	141.2 ± 0.2
pH control with 0.1 M phosphate buffer	59.5 ± 0.1	131.9 ± 0.2
pH control with 0.2 M phosphate buffer	61.0 ± 0.1	135.2 ± 0.2
pH control with 0.5 M phosphate buffer	61.2 ± 0.2	135.7 ± 0.4

[a] Means of the determinations with standard deviation $n = 3$. [b] The method of Desgranges et al. (1991) was used as a reference for recovery calculations.

solutions was used as a standard reference for the estimation of glucosamine concentration. The obtained linear regression equation was the following: y (absorbance in optical density unit) = $0.344x$ (glucosamine concentration, mg/mL). Since color intensity might be interfered with by coexisting substances in the solutions (Tsuji et al., 1969; Plassard et al., 1982; Desgranges et al., 1991), such as proteins, absorbance should be read against a blank containing all ingredients except glucosamine when subjected to quantitation.

In this study, we attempted to develop a one-step hydrolysis of chitin to release glucosamine for colorimetric quantitation; this has been based largely on the two-step hydrolysis method reported by Desgranges et al. (1991). Some essential findings have been shown in Table 1. By the two-step hydrolysis method, the glucosamine content was 45.1% and was used as a reference for estimation of the relative glucosamine recovery. Prior to HCl hydrolysis, vacuum treatment of the chitin sample in 10 M HCl enhanced hydrolysis efficiency. When the untreated sample was hydrolyzed at 140 °C for 60 min, the relative glucosamine recovery was 79.4%. However, when the sample was vacuum-treated prior to hydrolysis at 140 °C for 60 min, the relative glucosamine recovery was 135.9% in proportion to the recovery obtained from the two-step hydrolysis method. This reveals that the one-step method is better than the two-step method in glucosamine recovery. On the aspect of timing, time of heating for chitin hydrolysis was important. Under an optimized conditions, chitin can be hydrolyzed with acid to cleave the $\beta(1-4)$ glycosidic bonds, remove the acetyl group, and produce glucosamine for colorimetric quantitation. However, excessive acid treatment may result in the breakdown of glucosamine and a decrease of recovery. In Table 1, the relative recoveries of glucosamine increased with time from 30 to 90 min. On the basis of the fact that relative glucosamine recovery increased remarkably from 30 min to 60 min and then increased slightly to 90 min, 60 min of thermal hydrolysis was practiced in this study. In the literature, a comparatively longer time than 60 min is required for chitin hydrolysis (Ride and Drysdale, 1971, 1972; Bethlenfalvay et al., 1981; Holan et al., 1980; Plassard et al., 1982; Swift, 1973; Cousin et al., 1984; Wu and Stahmann, 1975; Gurudiddaiah et al., 1978; Desgranges et al., 1991). Thus, vacuum treatment of the sample and acid hydrolysis for 60 min in a closed vessel at an elevated temperature is necessary.

When 2.5 mL aliquots of the chitin hydrolysate were combined with 15 mL of deionized water and subjected to pH adjustment with NaOH or a phosphate buffer (pH 12.5, 0.2 M) to pH 6.0, 7.0, or 8.0 followed by volume adjustment to 25 mL and a 10-fold dilution with deionized water, pH values of the buffered solutions did not change (Figure 2). Solutions adjusted to pH 8.0 with NaOH decreased in pH to 6.8 after a 10-fold dilution. When the diluted solutions were subjected to glucosamine analysis, glucosamine contents in the chitin samples observed in the phosphate-buffered solutions maintained a steady level at pH 6.0 and 7.0 and decreased slightly at 8.0 (Figure 2). However, for the nonbuffered cases, glucosamine contents decreased with an increase of pH adjusted by NaOH. When a series of phosphate buffer solutions with varied molarities at pH 12.5, that is, 0.1, 0.2, and 0.5 M, were applied for pH adjustment to pH 7.0, the glucosamine contents did not vary (Table 1). Therefore, the use of a phosphate buffer solution has provided a convenient means to control the pH value during serial dilutions and subsequent glucosamine quantitation.

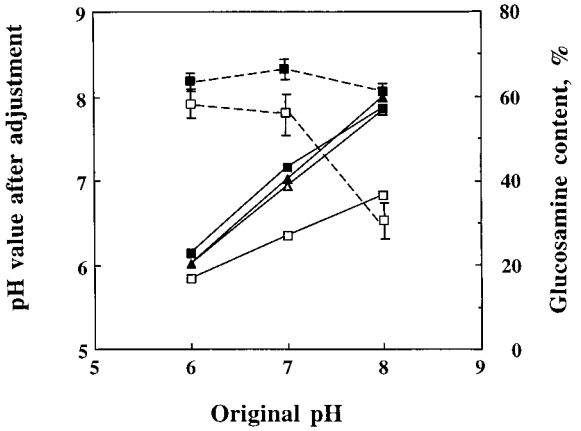

Figure 2. Changes in pH value and glucosamine content of 2.5 mL aliquots of a chitin hydrolysate solution while each was combined with 15 mL of deionized water and subjected to pH adjustment to pH 6.0, 7.0, and 8.0 with a phosphate buffer solution (buffered) or with NaOH (nonbuffered) followed by volume adjustment to 25 mL and a further 10-fold dilution with deionized water for colorimetric quantitation of glucosamine: (—) pH; (---) glucosamine content; (▲) buffered 25 mL solutions; (△) nonbuffered 25 mL solutions; (■) buffered and 10-fold diluted solutions; (□) nonbuffered and 10-fold diluted solutions.

When the modified one-step procedure for chitin hydrolysis in association with use of a phosphate buffer system was applied for glucosamine determination of various mold mycelia, average glucosamine content varied mainly depending upon mold species (Table 2). The influence of sample size ranging from 10 to 40 mg on the glucosamine content was minor. In comparison, the highest and the lowest chitin contents (based on

Table 2. Glucosamine Content in the Mycelial Mass of Various Molds As Affected by Sample Size

	glucosamine content, % (w/w)			
	sample size subjected to analysis, mg			
molds	10	20	40	average
Actinomucor tiwanensis	14.4 ± 1.4[a]	12.0 ± 0.6	12.3 ± 0.2	12.9 ± 1.4[b]
Aspergillus flavus	7.1 ± 0.5	8.2 ± 0.1	8.5 ± 0.2	7.9 ± 0.7
A. niger	16.5 ± 0.3	15.4 ± 0.1	14.5 ± 0.1	15.5 ± 0.9
A. oryzae	23.3 ± 0.6	27.0 ± 0.3	25.2 ± 0.4	25.2 ± 1.6
Penicillium citrinum	10.5 ± 1.7	11.7 ± 0.9	11.9 ± 0.7	11.4 ± 1.3
P. digitatum	19.5 ± 0.1	20.5 ± 0.1	16.5 ± 0.1	18.8 ± 1.7
Rhizopus microsporus	29.1 ± 1.7	28.7 ± 0.1	30.0 ± 0.1	29.3 ± 1.1

[a] Means of the determinations with range $n = 2$. [b] Means of the determinations with standard deviation $n = 6$.

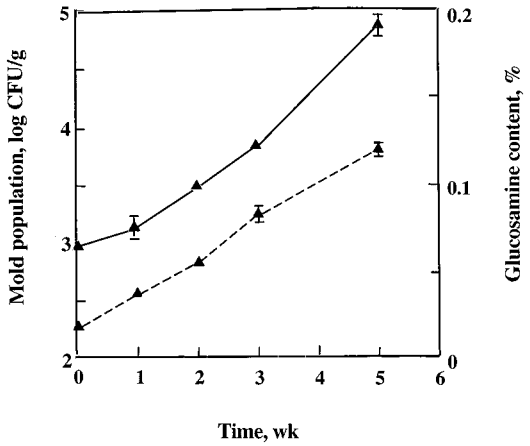

Figure 3. Changes in internal mold population (—) and glucosamine content (---) in the sound and mature peanut kernels during incubation under humidified conditions (RH 100% at 27 °C) for 5 weeks for natural mold contamination.

glucosamine content) were observed in the mycelia of *Rhizopus microsporus* and *A. flavus*, respectively. The chitin content of *Alternaria tenuis, Colletotrichum phomoides, Geotrichum candidum,* and *Fusarium oxysporum* varies depending upon cultural age, growth conditions, and species (Bishop et al., 1982). For those molds, the values ranged from 5.7 to 43 μg of glucosamine per milligram of dry mycelial weight. Cousin et al. (1984) analyzed six molds including *Alternaria alternata, Alternaria solani, Colletotrichum coccodes, Fusarium oxysporum, Geotrichum candidum,* and *Rhizopus stolonifer* and found that their chitin contents vary with dependence upon species and cultural age. Since the cultural age was identical for those test molds in this study, chitin content varied mainly depending upon mold species.

When peanut kernels were incubated under humidified conditions for 5 weeks for natural mold contamination, the kernels were periodically sampled and subjected to enumeration of internal mold population and determination of glucosamine content (Figure 3). Logarithms of the internal mold populations and glucosamine contents increased with an increase of incubation time. The increase of the former was more rapid than was the latter. When cooked rice and soybean were inoculated with conidia of *A. oryzae*, incubated for koji preparation, and subjected to analyses of mold growth (Figure 4), logarithms of the internal mold populations

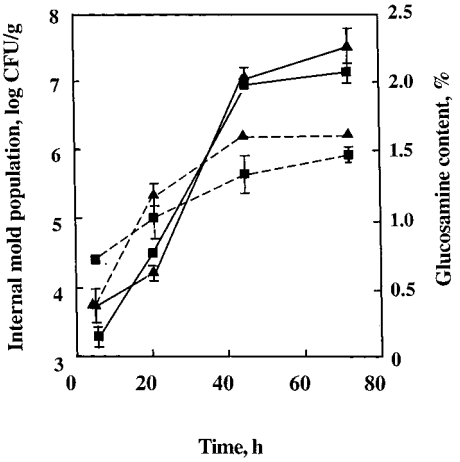

Figure 4. Changes in internal mold population (—) and glucosamine content (---) in the cooked rice (▲) and soybean (■) respectively inoculated with conidia of *Aspergillus oryzae* and incubated at 26−29 °C for 72 h for koji preparation.

and glucosamine contents both increased rapidly with an increase of incubation time. This was in agreement with the observation of Sakurai et al. (1977), who have demonstrated that glucosamine quantitation is convenient for the estimation of mold growth in koji. Donald and Mirocha (1977) have also determined chitin content as a measure of fungal growth in stored corn and soybean seed. Since the inoculum level during koji making was high (ca. 7 log CFU/g) and incubated at enhanced conditions, a rapid mold growth in the koji substrates was expected. Even both internal mold population and glucosamine content were available for estimation of mold growth in koji; the latter took less time than the former. In comparison to peanut kernels (Figure 3), lower internal mold populations and glucosamine contents than those of koji were obtained. Since the dry peanut kernels were not artificially inoculated and incubated under humidified conditions, moisture uptake by the kernels and growth of the natural mold contaminants developed slowly (Chiou, 1997). Nevertheless, the results have shown that the monitored procedure was appropriate for glucosamine quantitation in various substrates with various extents of mold infection.

In conclusion, a one-step 60 min HCl hydrolysis of chitin to release glucosamine for quantitation was achieved. A phosphate buffer (pH 12.5, 0.2 M) was effective in pH stabilization and enhancement of colorimetric determination of glucosamine content. When the modified procedure was applied to determine glucosamine (chitin) content in the mycelia of various molds, chitin content varied mainly depending upon mold species. When internal mold population and glucosamine content in peanut kernels incubated under humidified conditions for 5 weeks and cooked rice and soybean inoculated with conidia of *A. oryzae* and incubated for koji preparation were determined, logarithms of the internal mold populations and glucosamine contents both increased with increases in incubation time. The modified procedure provided a rapid and reliable means to estimate mold growth in various substrates. From the viewpoint of food safety, the procedure may be practical in the determination of the presence of chitin-containing materials such as tissues of fungi or insects contaminated in various foods. In particular, it has potential significance regarding spe-

cific mold contamination and resultant mycotoxin production in various grain products.

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