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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Glycated proteins: Clinical utility and analytical approaches

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The binding of glucose to serum proteins in a non-enzymatic reaction occurs proportionately to the prevailing serum glucose concentration during the lifespan of each protein. The non-enzymatic reaction of proteins with reducing sugars results in the formation of glycated proteins that are indicative of the relative amount of reducing sugars present. Hemoglobin, albumin, lipoproteins and other tissue proteins can be non-enzymatically glycated. Hence, estimation of these glycated proteins can be used for the estimation of average glycemic status. Spectrophotometric, chromatographic and immunoassays techniques are used for the detection and quantitation of these glycated proteins.

Key words: Glycated protein, reducing sugars, detection, quantitation.

INTRODUCTION

Protein glycation is a non-enzymatic process initially discovered in 1990s by Louis Camille Maillard who observed chemical modifications to amino acids when heated in the presence of reducing sugars, a process often referred to as the Maillard reaction (Maillard, 1912). This non-enzymatic glycation is a posttranslational modification in which a reducing sugar condenses with protein amino groups at the N-terminus or on lysyl side chains. The reaction is initiated with the attachment of the aldehyde or ketone carbonyl group of the acyclic saccharide to a free amino group in the protein, forming a Schiff base. Such an aldimine intermediate undergoes an amadori rearrangement to form 1-amino-1-deoxyfructose derivative forming a stable ketoamine linkage, which in turn can be cyclized to a ring structure (Lacinová et al., 2010). The cyclic amadori product then undergoes multiple dehydration and rearrangements, leading to the formation of advanced glycation end products (AGEs) (Nakayama et al., 1999). The formation of AGE further can lead to metabolic syndrome, clustering of several metabolic abnormalities (Miller and Adeli, 2008). This metabolic complication has been indicated to arise due to different factors including the use of antiretroviral treatment in HIV patients (Tesfaye et al., 2014).

Non-enzymatic glycation follows mass action kinetics, where by the amount of glycated product is proportional to the concentration of reactants, of which the ambient glucose concentration is a major determinant of the

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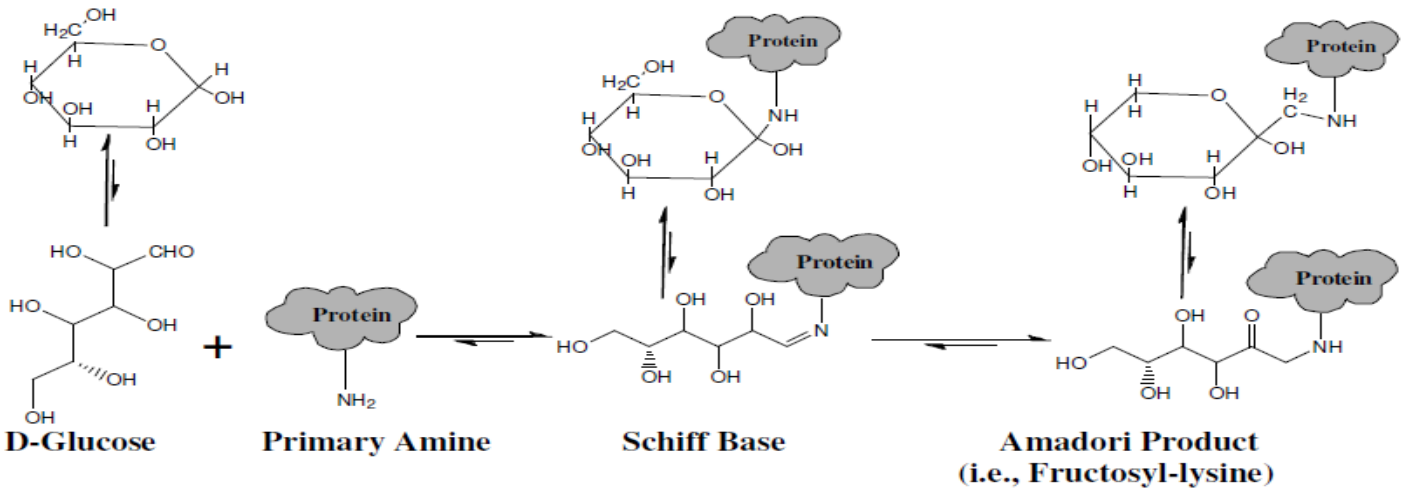

Figure 1. Chemistry of protein glycation adopted from characterization of glycation sites on human serum albumin using mass spectrometry: PhD Dissertation by Omar St. Aubyn Barnaby Graduate College at the University of Nebraska, 2010.

forward reaction rate. The second main determinant is the duration of a protein's exposure to an elevated glucose concentration, which relates to both its residence time in the circulation and episodes of hyperglycemia (Schleicher and Wieland, 1986). Hyperglycemia is associated with a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to diabetes mellitus with disturbances of carbohydrate, fat and protein metabolism (Megerssa et al., 2013).

In vivo, the amount of any glycated protein maintained in a steady state and is influenced by time-averaged glycemia, the rate of glycation, and the rate of the protein's degradation or removal from the circulation. During the process of glycation (Figure 1), glucose adducts can continue to form until equilibrium is reached, and a new population of the protein is subjected to the pertinent reaction kinetics as an older population is physiologically removed (Schleicher and Wieland, 1986). In persistent hyperglycemic patients, hemoglobin (Hgb), albumin, lipoproteins and other tissue proteins are non-enzymatically glycated to a great extent and are hall marks of diabetes mellitus. Hence, glycated hemoglobin (GHb) and glycated albumin (fructosamide) are widely accepted as reliable indicators of metabolic control in diabetic patients (Kobayashl et al., 1990).

CLINICALLY RELEVANT GLYCATED PROTEINS

Glycated hemoglobin

In adults, Hgb is usually constituted of HgbA (97%), $HgbA_2$ (2.5%) and Hgb F (0.5%). HgbA, in turn, is made up of four polypeptide chains, two α and two β chains (Brutis et al., 2006). Glycation occurs at several amino acid residues of the different variants of hemoglobin (HgbA, $HgbA_2$ and HgbF) and results in a product called glycated hemoglobin (GHb). Chromatographic analysis of HgbA reveals a number of minor species $HgbA_{1a}$, $HgbA_{1b}$ and $HgbA_{1c}$ that are collectively known as fast hemoglobin. $HgbA_{1c}$ is the specific amadori product of glucose conjugated with valine at the N-terminal of both β chains of HgbA. This product accounts for approximately 80% of $HgbA_1$ and about half of the total GHb (Benjamin and Sacks, 1994). Other glycohemoglobin species products have glucose linked to an ϵ-amino group of one or more of their lysine residues on their α or β chain (Cohen and Clements, 1999).

Formation of GHb is essentially irreversible and its concentration in blood depends on both the life span of red cells and blood glucose concentration. As the rate of formation of GHb is directly proportional to the concentration of glucose in the blood, the GHb concentration represents the integrated values for glucose in the preceding 6 to 8 weeks. This provides an additional criterion for assessing glucose control because GHb values are free of day-to-day glucose fluctuations and are unaffected by recent exercise and food ingestion (Brutis et al., 2006).

Clinical use of HbA_{1c}

HbA_{1c} level reflects the blood glucose level consistent with the live span of red blood cell, which is close to 120 days (Nitin, 2010). Hence, higher levels of HbA_{1c} are found in people with persistently elevated blood sugar, as in diabetes mellitus. However, though HbA_{1c} is claimed to

reflect the weighted glucose value over for 120 days, practically it is more inclined to show the glucose values in the weeks close to blood collection. In more detail, it can be said that the mean glycemia during the month preceding the HbA_{1c} measurement contributes 50% of the result, whereas the glucose value during the 30-60 days prior to the HbA_{1c} accounts for the 25% and the remaining 25% reflects the glucose level during the whole 60-120 days prior to the measurement. The approximate mapping between HbA_{1c} values and estimated average glucose (eAG) measurements is given by the following equation: eAG (mg/dl) = 28.7 × HbA_{1c} − 46.7or eAG(mmol/l) = 1.59 × HbA_{1c} − 2.59 (Sultanpur and Kumar, 2010). In general, high values of HbA_{1c} are used to assesses the risk of diabetes complications in addition to the diagnosis of diabetes mellitus. Other applications of HbA1c include prediction of cardiovascular events, even in individuals without diabetes, fasting hyperglycemia and metabolic syndrome (Reddy et al., 2012).

Limitation of the $HbA1_c$ measurement

Any condition that shortens erythrocyte survival or decreases the mean erythrocyte lifespan (e.g., recovery from acute blood loss, hemolytic anemia) falsely lowers GHb test results regardless of the assay method. Drugs such as aspirin are also reported to falsely lower test results, possibly by inhibiting glycation of Hgb (Reddy et al., 2012). Because human body replaces the red blood cells and the Hgb they contain every 120 days, the HbA_{1c} test cannot be performed more frequently than this 120 day time frame and yield a meaningful result. In addition, the HbA_{1c} test does not directly measure the serum protein glycation (Epinex, 2008).

Methods for measurement of glycated hemoglobin

Specimen for measurement of glycated Hgb: There are a number of methods to measure GHb including chemical method, high performance liquid chromate-graphy, affinity chromatography and immunoassay. All of these methods require only very small volumes of red cells and there are no special collection conditions. Furthermore, there is no need for fasting before sample collection. The anticoagulant ethylenediaminetetraacetic acid (EDTA) is preferred to collect blood but heparin and fluoride oxalate may also be used (John, 2003).

Method based on chemical reactivity: Chemical method of GHb estimation is based on generation of 5-hydroxymethylfurfural (5HMF) from glycol amino groups on Hgb, by heating the GHb in a weak acid. This chemical method measures the total glycated Hgb, that is, it measures not only HbA_{1c}, which is glycated at its N-terminal residue, but also Hgb variants that are glycated at sites other than their N-terminal site. Then, the 5HMF generated is reacted with thiobarbituric acid and quantified colorimetrically. Therefore, method estimates HbA_{1c} as well as HbA_{1a} and HbA_{1b}, and hence, the values obtained are higher than chromatography by 1-2% (Chandalia and Krishnswamy, 2002).

High-performance liquid chromatography (HPLC): In this technique, the Hgb solution is treated with endo-proteinase Glu-C in order to hydrolyze its constituent proteins into several peptides, among them are the glycated (HbA_{1c}) and the non-glycated (HbA_0) N-terminal hexapeptides of the β-chains. Thereafter, the resulting glycated and non-glycated N terminal hexapeptides of the β-chains are separated from the crude peptide mixture and quantified by HPLC and electrospray mass spectrometry or by HPLC followed by capillary electrophoresis with UV detection. The percentage of HbA_{1c} is determined as a ratio of the glycated to non-glycated β-N-terminal hexapeptides of Hgb (Jeppsson et al., 2002).

Affinity chromatography: Affinity chromatography is a method of separating biochemical mixtures based on specific interaction between receptor and ligand. The method utilizes interaction between the 1,2-cis diol group of GHb and immobilized boronic anions in a column, then separation of GHb from the column is achieved using buffers. First, the non-glycated Hgb elutes directly from the column together with the first buffer; after it the bound hemoglobin, GHb, dissociate by the use of a counter ligand (e.g. Sorbitol). The absorbance of the Hgb fractions can be measured at 414 nm using spectro-photometer, and the percentage of GHb is calculated (John, 2003).

Immunoassay: This method utilizes a monoclonal antibody raised against HbA_{1c}, which recognizes the first eight amino acids on the β chain Hgb together with the attached glucose. For the reaction to proceed, first red cells in a sample are haemolysed, the Hgb oxidized and the pH adjusted to optimize binding to a microtitre plate. Then monoclonal antibody-enzyme conjugate bound to the HbA_{1c} is immobilized on the plate while any unbound conjugate is removed by washing. Horseradish peroxidase in the bound conjugate normally reacts with the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and produces a color. The reaction was then stopped with acid and the color intensity was measured at 450 nm using a microtitre

plate reader. Finally, readings were made by relating to calibrators containing known percentage of HbA_{1c} (John, 2003).

Fructosamine

The term “fructosamine” refers to the sum of all ketoamine linkages between circulating glucose and serum proteins. This substance does not contain fructose; however, the glucose chain, which is linked to the protein amino group in a ketoamine bond, becomes modified to resemble the open-chain form of fructose. All serum proteins can become glycated, and therefore, all glycated serum proteins are fructosamines. Glycated albumin accounts for approximately 80% of measured serum fructosamine (Klonoff, 2000). Serum fructosamine represents an index of intermediate glycaemic state (2-3 weeks) that alert physicians to deteriorating or even improvement in their glycaemic control before that of GHb. However, as it represents glycation of protein (mainly albumin), its value is mainly affected by serum protein concentration and the half-life of the proteins both in normal individuals, and in patients with altered protein metabolism (Mula-Abed and Hanna, 2001).

Assay for fructosamine

There are several methods developed for the determination of fructosamine. A procedure using furosine and HPLC is accepted as the reference method. However, a colorimetric procedure using nitrobluetetrazolium (NBT) has gained popularity due to its speed, reproducibility and ease of automation (Scleicher and Vogt, 1990). This assay suffers from interferences due to uric acid and lipemia. The modified colorimetric method that utilizes addition of uricase and detergent, to remove uric acid and lipemia agrees well with fructosamine determined by use of furosine/HPLC method.

NBT utilizes the principle that fructosamines are reductants under alkaline conditions. Hence, in the presence of the NBT, fructosamines are reduced to formazae, a color product, which can be measured spectrophotometrically. The results are expressed as mmol/L of desoxymorpholino-fructose (DMF), which is the synthetic ketoamine, used as primary standard (Johnson, 1983).

Nevertheless, in the NBT method, the fructosamine concentration falsely increases due to some reducing components contained in serum, e.g., thiol group in the peptides and proteins. These reducing components mainly reacted with NBT pre-incubation of the serum sample to avoid this interference resulting from thiol group which could not be undetected throughout the assay (Xua et al., 2002).

Glycated albumin

Glycated albumin refers to albumin to which glucose has bonded. It usually accounts for 80% of the glycated serum proteins. Its circulating half-life is about 17 days, which is shorter than that of the average red cell life of 120 days. This feature makes periodic determination of glycated albumin an attractive candidate for assessing short to intermediate-term level of the average glucose level (Kumeda et al., 2008).

Chemistry of albumin glycation

Non-enzymatic glycation of albumin occurs at multiple sites. Glucose can attach at Lysine 199, Lysine 281, Lysine 439 and Lysine 525, other minor lysine and arginine residues, and also at N-terminal residues of polypeptides. Lysine 525 accounts for 33% of all glycation. Initial reactions are reversible, but subsequent reactions involving protein unfolding and refolding give rise to irreversible cross-linked rearranged products of glucose with proteins. This results in a stable form of glycated albumin that persists at markedly elevated levels in the plasma of diabetic patient (Iberg and Fluckiger, 1986).

Clinical utility of glycated albumin

Glycated albumin has been recently used as another clinical indicator of glycemic control. It provides a short-term index of glycaemic control, and it is not influenced by albumin concentration (Schleicher et al., 1993). In addition, glycated albumin is not affected by RBC lifespan (Inaba et al., 2007). In diabetic subjects, glycated albumin has strong correlations with glucose and provides a reliable index of glycaemic control over the preceding 2–3 weeks (Tahara and Shima, 1995). Glycated albumin concentrations increase and decrease more rapidly with fluctuations in overall glucose as compared to HbA_{1c} and this allows rapid changes to be detected at an earlier stage (Takahashi et al., 2007). It has been revealed that increased levels of glycated albumin are linked to both the presence and severity of cardiovascular disease, and impaired renal function (Pu et al., 2007). Observations of the biological properties of glycated albumin are also related to the pathogenesis of diabetic vascular complications (Amore et al., 2004). Furthermore, glycated albumin reflects endogenous insulin secretion more sensitively than HbA_{1c} (Koga et al., 2010).

Clinical implications of glycated albumin

Glycation of albumin significantly reduces transport ability of albumin because of the conformational change associated with glycation that causes a decrease in binding affinity. As compared to the non glycated form, the affinity of glycated albumin to bilirubin is reduced by 50% and is also reduced by 20-fold for the long chain fatty acid, *cis*-paranaric acid. This means that the glycation of serum albumin will have a negative impact on the transportation of serum molecules (Epinex, 2008). In addition, glycation of albumin results in impairment of its protein structure and consequently its antioxidant properties (Singh et al., 2007).

Macrophages in the artery walls recognize glycated albumin via specific receptors and, in turn, trigger activation of endoplasmic reticulum kinase, a potent cell-signaling pathway that activates nuclear factor kappa B (NF- KB), a key player in inflammatory reactions. This also produces potent cytokines like transforming growth factor beta (TGF β), the corollary being a perpetuation of the inflame-matory pathways in the artery wall that charac-terizes the evolution of the atheroma plaque (Hattori et al., 2002).

Diabetic nephropathy can also be generated by the interaction of glycated albumin with receptors in the mesangial cells, independently of the direct actions of hyperglycemia through an amplification cell signaling cascade, involving protein kinase C and secretion of potent cytokine like TGF-β, a series of deleterious effects occur that produce glomerular dysfunction and albuminuria (Epinex, 2008).

Glycated albumin stimulates the phosphorylation of c-Jun, a component of the transcriptional factor activation protein-1 (AP-1) in retinal glial cells. AP-1 up regulates the mRNA level of cytokine vascular endothelial growth factor (VEGF), stimulating increased levels of VEGF, and proliferation of unregulated capillary growth. When the newly formed capillaries invade the retina, leakage of blood plasma damages the retinal area, inducing macular degeneration. The result is a loss of vision in the central retinal area (Okumura et al., 2007).

Measurement of glycated albumin

The concentrations of glycated albumin can be measured by chromatography, thiobarbituric acid assay and immunoassay. However, these methods have a number of disadvantages, e.g. specimens must be pretreated and procedures are complicated. In order to determine glycated albumin more easily and rapidly, a new enzymatic method or glycated albumin using albumin-specific proteinase, ketoamine oxidase and albumin assay is developed (Kouzuma et al., 2002). The glycated albumin is hydrolyzed to glycated amino acids by proteinase digestion, and ketoamine oxidase oxidizes the glycated amino acids to produce hydrogen peroxide, which is quantitatively measured. Glycated albumin value is calculated as the percentage of glycated albumin in total albumin, which is measured by the bromocresol green (BCG) method (Kouzuma et al., 2004).

Glycated lipoproteins

Lipids are important structural and bioregulatory com-ponents of human cells, and are transported in blood by the form of lipoproteins (Deric et al., 2006). Like other serum proteins, lipoproteins are non-enzymatically glycated in the presence of glucose (during normal aging and at accelerated rate in diabetes mellitus). Oxidation, accom-panying glycation *in vivo*, further supports chemical modifications (Graier et al., 1997).

Glycation of LDL occurs under hyperglycemic condi-tions in a time and a glucose concentration dependent way and favors the oxidation of LDL (Figure 2) (Galle and Wanner, 1999). Glycoxidation consists of two related processes oxidation of protein-bound sugars and oxidation of free glucose and its products. Both processes can generate radicals that modify LDL, and hence potentially contribute to the enhanced uptake of such particles by macrophage (Brown et al., 2007). These macrophages, then transform to so called foam cells, the lipid laden cells characteristic for an early atherosclerotic lesion (Deric et al., 2006). Glycation of HDL, which often accompanies hyperglycemic conditions, is associated with decreased paraoxonase levels, and renders HDL more susceptible to oxidation (Hedrick et al., 2000). Oxidized HDL has impaired ability to promote cholesterol efflux, and adverse effects on vascular inflammation (Ansell et al., 2007).

Clinical utility of glycated LDL

LDL glycation is likely to reflect blood sugar variations over a much shorter period. This is because the plasma half-life of LDL is only about 3 days, meaning that the breakdown of glycated protein and the production of fresh unglycated protein occur at a faster rate in the case of LDL, thereby, diluting the glycated LDL and probably minimizing the effects of blood sugar changes that occurred more than 3 days before measurement. Glycated LDL could also be particularly suitable as a measure of cardiovascular risk because it initiates and promotes several critical pathological processes that are involved in vascular disease. An additional advantage of its short term variability is that it could be useful for titrating the dose of glucose lowering treatment over a few days, thereby, allowing rapid control of hyperglycemia (Veiraia

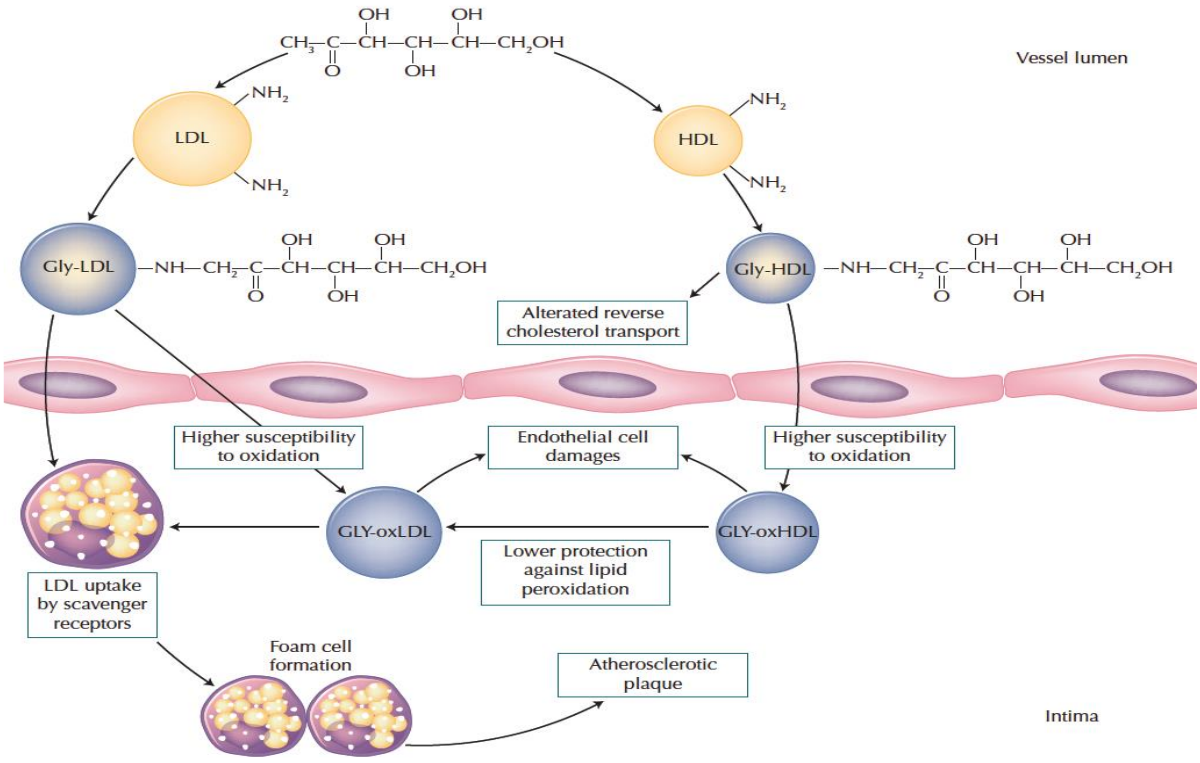

Figure 2. Glycation of HDL and LDL adopted from current atherosclerosis reports, 2007; 9:57-63.

and Wales, 2005).

Analytical approach for glycated lipoproteins

Analysis of glycated lipoproteins (glycated LDL and HDL) involves use of HPLC with an affinity boronate column and a gel permeation column. The system consists of three processes. In the first, serum proteins are resolved into glycated proteins and non-glycated proteins by affinity chromatography with the boronate column. In the second, glycated and non glycated proteins are separately resolved into glycated and non-glycated lipoproteins (LDL and HDL) with the gel permeation column. In the last, the cholesterol content of glycated and non glycated lipoproteins (LDL and HDL) is measured enzymatically. These three processes are automatically regulated by a system controller. By this method, glycated lipoproteins (glycated LDL and HDL) in a small serum sample can be measured easily within a short period of time (Tanaka, 2001). Glycated LDL and glycated HDL are presented as a percentage of total LDL and total HDL, respectively. The following formula is used to calculate their percentage: Glycated LDL (%) = (glycated LDL) / (glycated LDL + non-glycated LDL) x 100 and glycated HDL (%) = (glycated HDL) / (glycated HDL + non-glycated HDL)] x 100 (Tanaka et al., 2006).

CONCLUSION

Glucose binds to serum proteins in a non-enzymatic reaction, which occurs in proportion to the prevailing serum glucose concentration during the lifespan of each protein. This non enzymatic reaction is known as glycation, and the product is called glycated protein. Important glycated protein products include: GHb, fructosamine, glycated albumin and glycated lipoproteins. Glycation of proteins affords an index for glycemic control. Moreover, literatures reported that increased glycated proteins are linked to nephropathy, cardiovascular diseases, retinopathy, etc. Several methods such as spectrophotometry, chromatography and immunoassays are presently employed for quantification of different glycated proteins.

Conflict of interest

The authors did not have any conflict of interest.

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Full Length Research Paper

Characterization of partially purified cysteine protease inhibitor from *Tetracarpidium conophorum* (African walnut)

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Cysteine protease inhibitors (CPIs) have been known to be present in a variety of seeds of plants, and have been intensively studied as useful tools for potential utilization in pharmacology. This study reports the isolation of CPI from *Tetracarpidium conophorum* by 65% ammonium sulphate saturation, followed by ion exchange chromatography; further purification was by gel filtration chromatography. The molecular weight of the partially purified protein inhibitor was analyzed by SDS-PAGE to be approximately 20 kDa. The inhibitor had an optimum pH and temperature of 8.0 and 40°C, respectively. The inhibitor competitively inhibited papain with the same V_{max} = 71.17×10^3 µmol/min, K_m = 166 µM, and K_i = 53.63 µM. Divalent metal ions such as, Mg^{2+}, Pb^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} had significant effect on inhibitory activity of CPI at concentration as low as 1 mM. Cysteine protease inhibitor of *T. conophorum* investigated in this study could serve as a template in biotechnology of herbal medicine to arrest the negative modulatory interactions of cysteine proteases in clinical pathogenic expressions.

Key words: *Tetracarpidium conophorum,* cysteine protease inhibitor, papain, purification, characterization.

INTRODUCTION

The interaction between cysteine proteases and their inhibitors is a requisite in preventing unwanted, potentially destructive proteolysis, which can be utilized in chemo-therapy. Cysteine proteases (CPs) are widely distributed among living organisms, and are responsible for many biochemical processes occurring in living organisms, such as production of nutrients for cell growth and proliferation in *Rhizopus oligosoporus* (Lin et al., 2011). They play multi-faceted roles in plants, virtually in every aspect of their physiology and development, such as growth, senescence and apoptosis, in the accumu-lation and mobilization of storage proteins such as in seeds. They are also involved in signaling pathways triggered by protease-activated receptors (PARs), which are critical mediators of haemostasis, thrombosis, biotic and abiotic stresses, and have been implicated in cancer progressions, making this receptor class an important drug target (Chen et al., 2005). The *Carica papaya* enzyme papain (EC 3.4.22.2) was the first known proteolytic enzyme and its digestive properties were already being utilized in the

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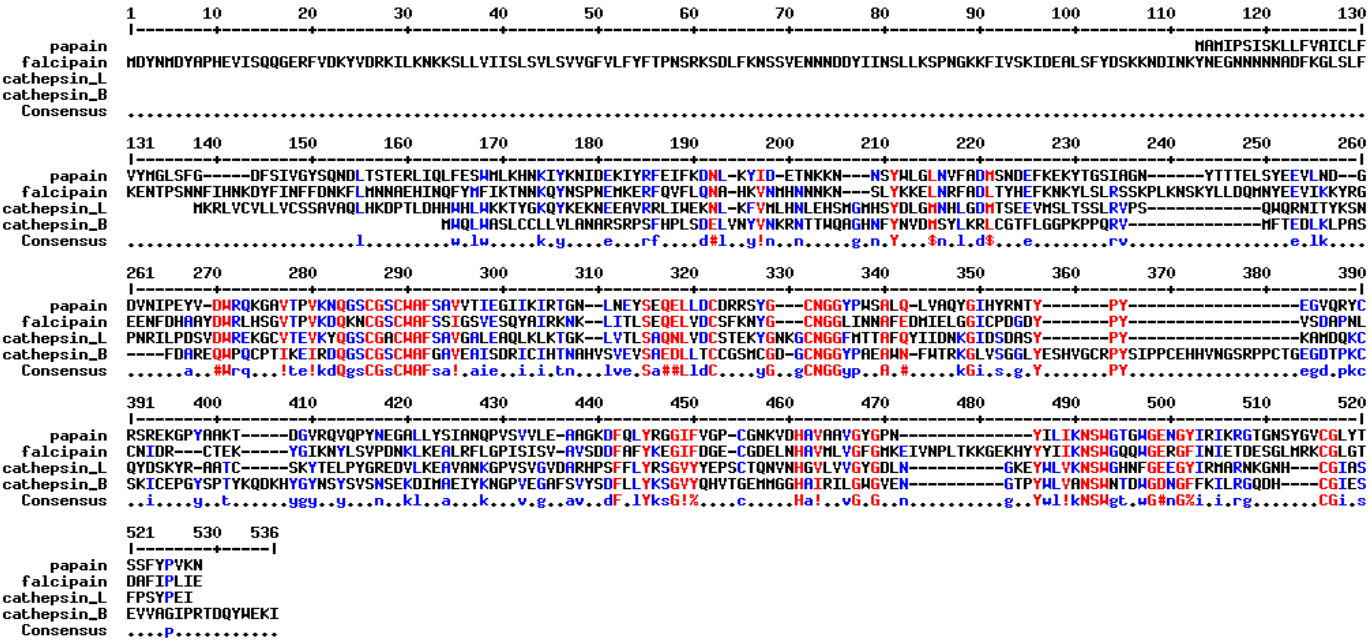

Figure 1. Sequence alignment of structurally equivalent residues of some C1 family of cysteine proteases. Coloured red are residues that are conserved; blue are similar residues and black are residues that are different in all the groups.

19th century (Redzynia et al., 2009). Since then papain has been used as a model enzyme in many studies and is a founding member of a large C1 family of papain-like cysteine proteases (Rawlings et al., 2008), while calpain (EC 3.4.22.52) constitute the C2 family of calcium-dependent non-lysosomal cysteine proteases expressed ubiquitously in mammals and many other organisms (Ohno et al., 1984). C1 family of proteases are evolutionarily old and are found in both prokaryotes and eukaryotes and show activities that are indispensable for the organism (Redzynia et al., 2009). Some of the mammalian cysteine proteases are evolutionarily closely related to papain and hence belong to this family for example cathepsins B, H, L, S and K (Figure 1). These enzymes function in every cell as components of the lysosomal degradation system taking part in the turnover of proteins as well as participate in a number of proteolytic cleavages, activating pro-hormone and regulation of antigens (Redzynia et al., 2009).

Obligatorily, the activities of these enzymes need to be strictly regulated and controlled (Corrion et al., 2010) , by cysteine protease inhibitors. Activities of proteases are generally regulated by protease inhibitors, which are usually proteins with domains that enter or block a protease active site to prevent substrate access (Figure 2). A control mechanism of proteases involves interaction of the active enzymes with proteins that inhibit their activities. These proteins have been isolated and characterized from a large number of organisms, including plants, animals and microorganisms (Outchkourov et al., 2003). C1 family of cysteine proteases are in equilibrium with protein inhibitors belonging to the cystatin family (Hartmann et al., 1997; Rawlings and Barrett, 1990; Lustigman et al., 1992; Rawlings et al., 2008). Most cystatins such as human cystatin B are single domain proteins of 100-120 residues with a characteristic wedge-like epitope consisting of the N-terminus and two β-hairpin loops which blocks the active site cleft of the target enzyme hence inhibiting the activity in a reversible manner (Abrahamson et al., 1986; Bode et al., 1988) (Figure 2).

The African walnut is an annual agricultural product found abundantly in Nigeria (Ayoola et al., 2011b). The plant, which belongs to the family of *Euphorbiaceae*, is a temperate fruit and nut crop, with a high fat content hence Walnuts are rich source of energy and contain health benefiting nutrients, minerals, antioxidants and vitamins that are essential for optimum health (Ayoola et al., 2011a). Walnuts are considered to be an herb in traditional Chinese medicine (Ganiyu and Mofoluso, 2004). The leaves, bark, root and fruit of *Plukenetia conophora* are considered to be medicinal (Enitan et al., 2014). The anti-microbial potential of *P. conophora* extracts and fractions against wide spectrum of bacteria (including *Staphylococci*, *Clostridia*, *Escherichia* and *Pseudomonas*) and some fungi like *Aspergillus niger* and *Candida albican* have been demonstrated (Ajaiyeoba and Fadare, 2006). The ethanol: water extract of the dried leaves had the best antioxidant activity; the broad range of antioxidant activity of this extract indicates the potential of the plant as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress and consequent health benefits (Amaeze et

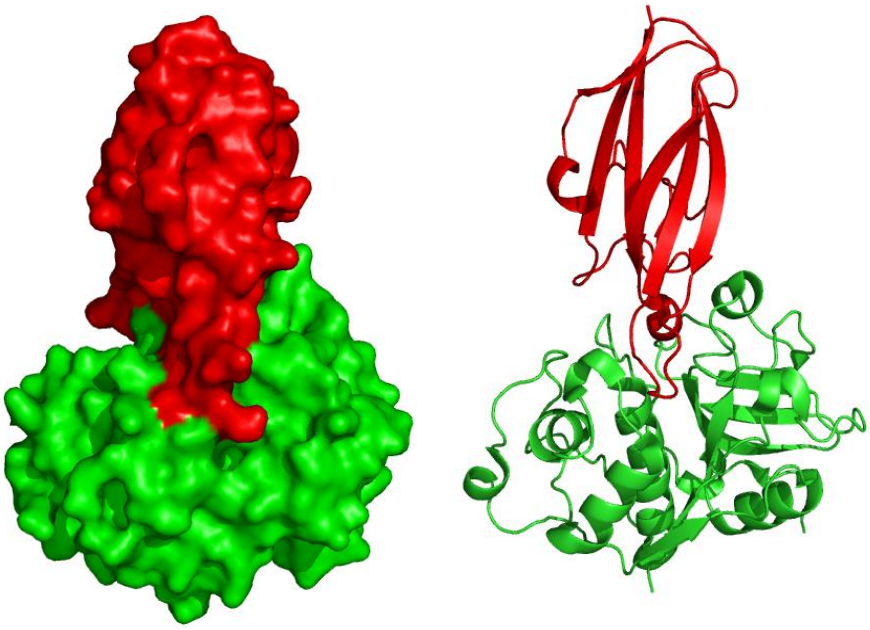

Figure 2. Surface and cartoon representations of the crystal structure of Chagasin-papain complex. The chagasin molecule is coloured in red and papain in green. The view is along the catalytic cleft of papain and corresponds to the standard orientation used for cysteine proteases (Redzynia et al., 2009; pdb: 3E1Z).

al., 2011). Antibacterial activity of methanolic leaf extract of *P. conophora* Mull. arg. against selected bacteria isolated from urinary tract infection has also been demonstrated (Enitan et al., 2014). The aim of the study was to isolate, purify and characterize cysteine protease inhibitor (CPI)from *Tetracarpidium conophorum* seeds.

MATERIALS AND METHODS

Sample collection and preparation

T. conophorum seeds and leaves were obtained at Ketu market in Lagos State, Southwestern Nigeria. The leaves were deposited at the herbarium of the Botany Department, Faculty of Science, Lagos State University, Ojo Lagos State, Nigeria for proper identification and authentication. 1000 g of *T. conophorum* seeds were de-shelled, dried and ground to powder with a grinding machine. The powdered sample was then defatted with n-hexane using the soxhlet apparatus, according to the method of Franz von Soxhlet (1879).

Extraction and isolation of cysteine protease inhibitor

Cysteine protease inhibitor was isolated as described by Benjakul et al., 1998. 40 g of the defatted sample was extracted in 500 ml ice-cold 100 mM phosphate buffer pH 7.2 containing 130 mM NaCl and 0.1% β-mercaptoethanol, stirred thoroughly for 1 h. The mixture was filtered using clean white piece of cloth. The filtrate was centrifuged at 7000 *g* for 10 min to collect cell debris. Cysteine protease inhibitor was then isolated from the resulting supernatant by ammonium sulphate precipitation according to the method described by Englard and Seifter (1990). Ammonium sulphate required to precipitate the protein was optimized by adding varying concentrations, (35, 55, 65, 75 and 90%) to the crude extract independently.

After each precipitation the precipitate was collected by centrifugation at 7000 *g* for 10 min. The precipitate was then re-dissolved in a small volume of buffer and dialyzed overnight against 100 mm Tris buffer pH 7.8 that was changed every 6 h.

Protein determination

Protein concentration was determined by Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard. The absorbance was read at 595 nm.

Inhibitory activity

The inhibitory activity of cysteine protease inhibitor on papain was monitored according to the modified method of Murachi, 1970, using casein as substrate. Papain (6 mg in 100 mM Tris-HCl buffer, pH 7.8, 0.5 mM cysteine, 0.2 mM EDTA) and the inhibitor extract (50 µl) were pre- incubated at 37°C for 15 min. The reaction mixture (150 µl) was then added to tubes containing 1.0 ml of 0.5% casein (casein prepared in 100 mM Tris-HCl pH 7.8 containing 0.2 mM EDTA and 0.5 mM cysteine) at 37°C. The assay was incubated for 30 min at 37°C and the reaction terminated by the addition of 1.0 ml of 5% trichloroacetic acid (TCA). The absorbance of the supernatant was measured at 280 nm after 30 min. One unit of inhibitor activity was defined as the decrease by one unit of absorbance of tricholoroacetic acid-soluble casein hydrolysis product liberated by protease action at 280 nm at 37°C in a given assay volume. Percentage Inhibition was determined as shown below:

Table 1. Purification table of cysteine protease inhibitor (CPI) from *T. conophorum.*

Sample	Total protein (mg)	Inhibitory activity (units)	Specific inhibitory activity (units/mg)	Protein yield (%)	Activity yield (%)	Purification fold
Crude extract	363	4000	11.02	100	100	1
Ammonium sulphate Fraction (65%)	126	1500	11.9	34.7	37.5	1.1
Ion Exchange chromatography (DEAE-cellulose)	46	3500	76.1	12.67	87.5	6.9
Gel filtration (Sephadex G-100)	26	3330	128.1	7.16	83.25	11.61

The purification fold of cysteine protease inhibitor from ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography were 1.1, 6.9 and 11.6 respectively.

$$\% \text{ Inhibition by each tube} = \frac{\text{Abs of Std} - \text{Abs of Sample}}{\text{Abs of Std}} \times 100$$

Protein purification

The crude protein after dialysis was purified by ion exchange chromatography according to the method of Rossomando (1990), followed by size exclusion chromatography. 3.0 ml of the dialysate (with highest % inhibitory activity against papain) was then loaded on DEAE-cellulose column previously equilibrated with 100 mM Tris-HCl buffer pH 7.8. 5 ml fractions were collected into 60 test tubes using an increasing linear gradient of NaCl concentration from 0 to 0.3 M in the same buffer (Tris-HCl). Total protein and inhibitor activity were carried out on each fraction as earlier described. Fractions with highest inhibitory activity were pooled together concentrated and loaded on sephadex G-100 column previously equilibrated with 100 mM Tris-HCl buffer pH 7.8 and eluted using the same buffer. Fractions with highest inhibitory activity were pooled together and analyzed further by SDS-PAGE for molecular weight estimation.

Mechanism of inhibition

Mode of inhibition of the purified CPI was carried out as described for inhibitory assay above but now varying the concentration of casein. This was carried out both in the absence and presence of the inhibitor, respectively.

Optimum temperature

The optimum temperature for the inhibitor was determined by incubating the reaction mixtures at varying temperatures ranging from 10 - 100°C with 10-unit increase.

Optimum pH

Estimation of the pH optimum was carried out using Tris-HCl buffer with varying pH (6-11) differently in the reaction mixtures. Other steps were as described for inhibitory assay.

Effect of metals on inhibitory activity

Effect of different metal ions on protease inhibitory activity was carried out by incubating the protease inhibitor with different concentrations of various metals ions for 30 min followed by measuring inhibitory activity as described above. The metals that were investigated included Mg^{2+},Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+},and Pb^{2+}. Each with concentration between 1 to 10 mM.

RESULTS AND DISCUSSION

Plant CPIs have been researched extensively, but this study however is the first at attempting to characterize cysteine protease inhibitor from *T. conophorum. T. conophorum* is a temperate fruit and nut crop which possess an elevated level of fat content. The seed is an annual agricultural product, which is found abundantly in Nigeria (Ayoola et al., 2011).

CPI was isolated by ammonium sulphate precipitation, using different concentrations of ammonium sulphate (35, 55, 65, 75 and 90%) and maximal inhibitory activity was observed at 65% saturation and this was used in further studies. The increase in hinbitory activity observed at 65% might be due to an increase in concentration of the salt ions having a greater charge density, thus increasing its ionic strength through the decline in solvent molecules being used to solvate the salt ions for optimum saturation, stressing the role of hydrophobic aggregation of proteins in molecular interaction.

Bijina et al. (2011), reported 30 - 60% ammonium sulphate saturation for *Moringa oleifera* protein. The protein was separated from the salt ions by dialysis, which allows ammonium sulphate salts and small meta-bolites to diffuse across a semi-permeable membrane. This step of purification yielded a low purification fold close to the crude protein, which suggests the presence of impurities (Table 1). Further purification of cysteine protease inhibitor by chromatographic methods, using DEAE cellulose an anion exchanger, yielded multiple peaks with a single peak of maximum inhibitory activity and a slight increase in purification fold (Table 1).

The single peak with the maximum inhibitory activity obtained after ion exchange chromatography was purified further by gel filtration yielded multiple peaks with two

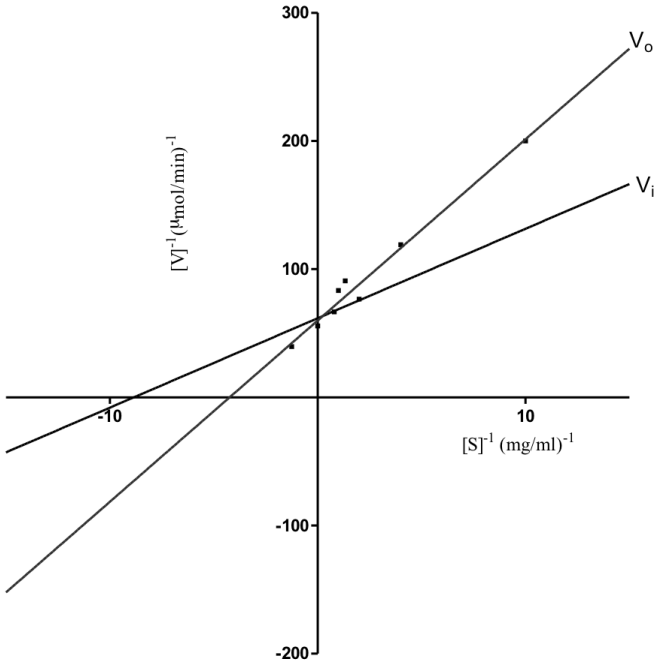

Figure 3. Line weaver-Burk plot of purified CPI. In the absence and presence of purified inhibitor extract, the reciprocal of the varying amount of substrate concentration [1/S] used and the reciprocal of the absorbance (1/V) at 280 nm were plotted against each other. The double reciprocal plot shows that inhibition is competitive having the same V_{max} of 71.17×10^3 µmol/min, different K_m =166 µM and K_i = 53.63 µM. $[V]^{-1}$ = with inhibitor and $[V_0]^{-1}$ = without inhibitor.

peaks having maximum inhibitory activity. This suggests that a splitting of the polypeptide chain might have occurred or there might be two different cysteine protease inhibitors present. The gel filtration chromatography using sephadex G-100, resulted in the highest purification fold of 11.61 and protein yield of 83.3% (Table 1). Bijina (2006), in his work, purified cysteine protease inhibitor from *Moringa oleifera,* using ammonium sulphate precipitation (30 - 90%) and ion exchange chromatography, and obtained purification fold of 1.5 and 2.5%, respectively. This could be accounted for by the fact that each purification step reduced the level of impurities associated with the inhibitor, which suggests that the fold can be increased by repetitive purification steps using advanced purification methods.

CPI from *T. conophorum,* demonstrated a competitive mode of inhibition on papain (Figure 3) with the same V_{max} = 71.17×10^3 µmol/min, K_m = 166 µM, and K_i = 53.63 µM. The result obtained was contrary to that reported by Bijina (2006), who reported the mechanism of protease inhibition of *Moringa oleifera* as uncompetitive. Kinetic studies between chagasin and papain (Figure 2) revealed the interaction to be a very strong reversible interaction with a K_i of 36 pM with chagasin blocking the active site cleft of papain (Redzynia et al., 2009) (Figure 2).

The protein inhibitor has an optimum pH of 8.0 (Figure 4) and observed to be inactive at extreme acidic and alkaline pHs. This indicates that extreme pH conditions could alter the electrostatic interactions between charged amino acids such as aspartate, lysine, arginine, and glutamate, thereby disrupting the structure of the cysteine protease inhibitor, making it loose its activity partially or completely. The optimum pH of cysteine protease inhibitors isolated in this study is in accordance with those reported for other isolated plants cysteine protease inhibitor such as *Dimorphandra mollis* seeds (pH 8.0) (Mello et al., 2002) and *M. oleifera* leaves (pH 7.0) (Bijina, 2006). *T. conophorum* CPI has a temperature optimum of 40°C (Figure 5). Most plant's cysteine protease inhibitors are active from this temperature up to 50°C (Bijina, 2006). However, the inhibitory activity declined at temperatures lower than its optimum temperature, and was totally inactive at temperatures above the optimum temperature.

The probable precursor for the modulating activities of cellular actino-regulatory proteases is a consequence of thermal influence on violently disrupted bonds of cysteine protease inhibitor from elevated kinetic energy (Bein and Simons, 2000). This result shows that cysteine protease inhibitor might undergo thermal denaturation when exposed to temperatures higher than its optimum temperature.

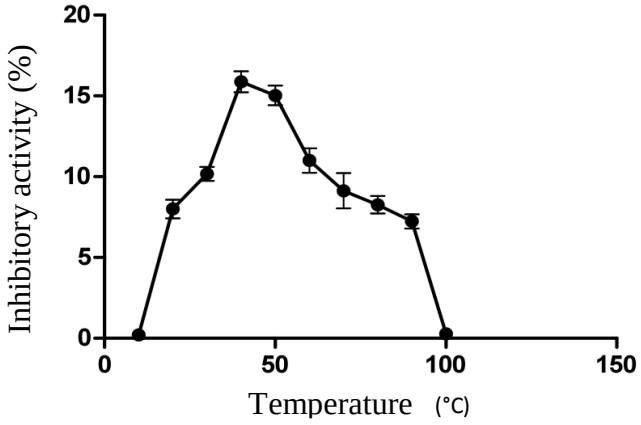

Figure 4. Optimum temperature of crude cysteine protease inhibitor. The result shows the optimal temperature of the cysteine protease inhibitor is 40°C. Cysteine protease inhibitor was almost inactive at low temperature, however as the temperature increases, the inhibitory activity increased gradually but decreased at temperatures above 40°C.

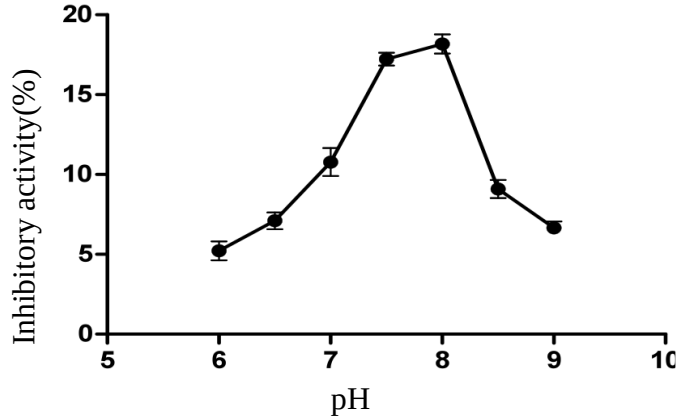

Figure 5. Optimum pH of purified cysteine protease inhibitor. The result shows the optimum pH of purified cysteine protease inhibitor at pH 8.0. Cysteine protease inhibitor was almost inactive at low pH values, however, as the pH increases, the inhibitory activity increased gradually but decreased at above pH 8.0.

This result is in agreement with those reported for other isolated plants cysteine protease inhibitor such as *Dimorphandra mollis* seeds (40°C) (Mello et al., 2002) and *M. oleifera* leaves (40°C) (Bijina, 2006).

Trace metal ions such as Co^{2+} and Zn^{2+} at low concentrations reduced the activity of CPI with a residual activity of 58 and 88%, respectively (Figure 6). However, when present in high concentrations, they are toxic and can denature proteins. Heavy metal ions such as Cu^{2+}, Mg^{2+}, Pb^{2+} and Mn^{2+} at 1 mm decreased the residual inhibitory activity of cysteine protease inhibitor up to 47.5, 51, 55 and 61.4%, respectively, compared to that of control. This suggest that these heavy metal ions interacted with the sulfhydryl groups of the cysteine protease inhibitor, thereby deactivating the inhibitor due to its sulphur-philic nature. The sulfhydryl group is respon-sible for the stabilization of tertiary and quaternary structure ofproteins. As a result, there is a conformational change in the three-dimensional structure of the protein and is denatured, thereby inhibiting their activity (Greenwood et al., 2002).

The heavy metal ions functioned in a concentration dependent manner, as their concentration increases, their inhibitory effect increases. The result obtained is in agreement with that of Jack et al. (2004), which confirmed that some divalent metal ions could play an important role in sustaining the structural integrity of protease inhibitors, such as Zn^{2+} and Mg^{2+}, that maintains the secondary and tertiary structure of cysteine protease inhibitor. The side chain carboxylates of glutamate and aspartate residues can however, participate in binding of divalent cations to metalloproteins, thereby resulting in amino acid modifica-tions of the structure leading to a decrease in inhibitory activity, whereby the inhibitor is unable to bind to the active site of the enzyme or substrate to exhibit inhibitory activity. Most metalloproteases require zinc, but some use cobalt (Szeto et al., 2008).

The purified CPI was further analyzed by SDS-PAGE to determine its molecular weight. The molecular weight of cysteine protease inhibitor from *T. conophorum* was estimated to be 20 kDa (Figure 7). This result indicates that *T. conophorum* CPI is composed of a single poly-peptide chain. Martinez-Vicente et al. (2005), reported a molecular weight of 23.1 kDa, for cysteine protease inhibitor obtained from *Fragaria x ananassa*. Cysteine protease inhibitor from black gram (*Vigna mungo Hepper)* and rice bean (*Vigna umbellata Thunb),* have molecular weight of 12 kDa by Tricine-SDS-PAGE (Benjakul and Visessanguan, 2000). Most of these protein inhibitors are small molecules with relative molecular masses ranging from 5 - 25 kDa (Singh and Rao, 2002; Lustigman et al., 1992).

The inhibition of papain and not trypsin, a serine protease (data not shown) reveals the specificity of this inhibitor against cysteine protease, which could be explored in biotechnology for the *in-vitro* synthesis and development of target drugs for pathogenesis of athero-sclerosis, dementia and cancer. The inhibition of calpain has been shown to prevent NMDA-induced cell death and β-amyloid-induced synaptic dysfunction in hippo-campal slice cultures (Nimmrich et al., 2010).

Conclusion

Cysteine protease inhibitor was isolated and partially purified from *T. conophorum*. The protein inhibitor was demonstrated to inhibit papain a representative of the C1 family of cysteine proteases in a competitive manner. The biological, specificity and physicochemical properties exhibited by this inhibitor certainly indicate its likely suitability for application in biotechnology as pharmaceutical agents in the treatment of some pathological conditions such as

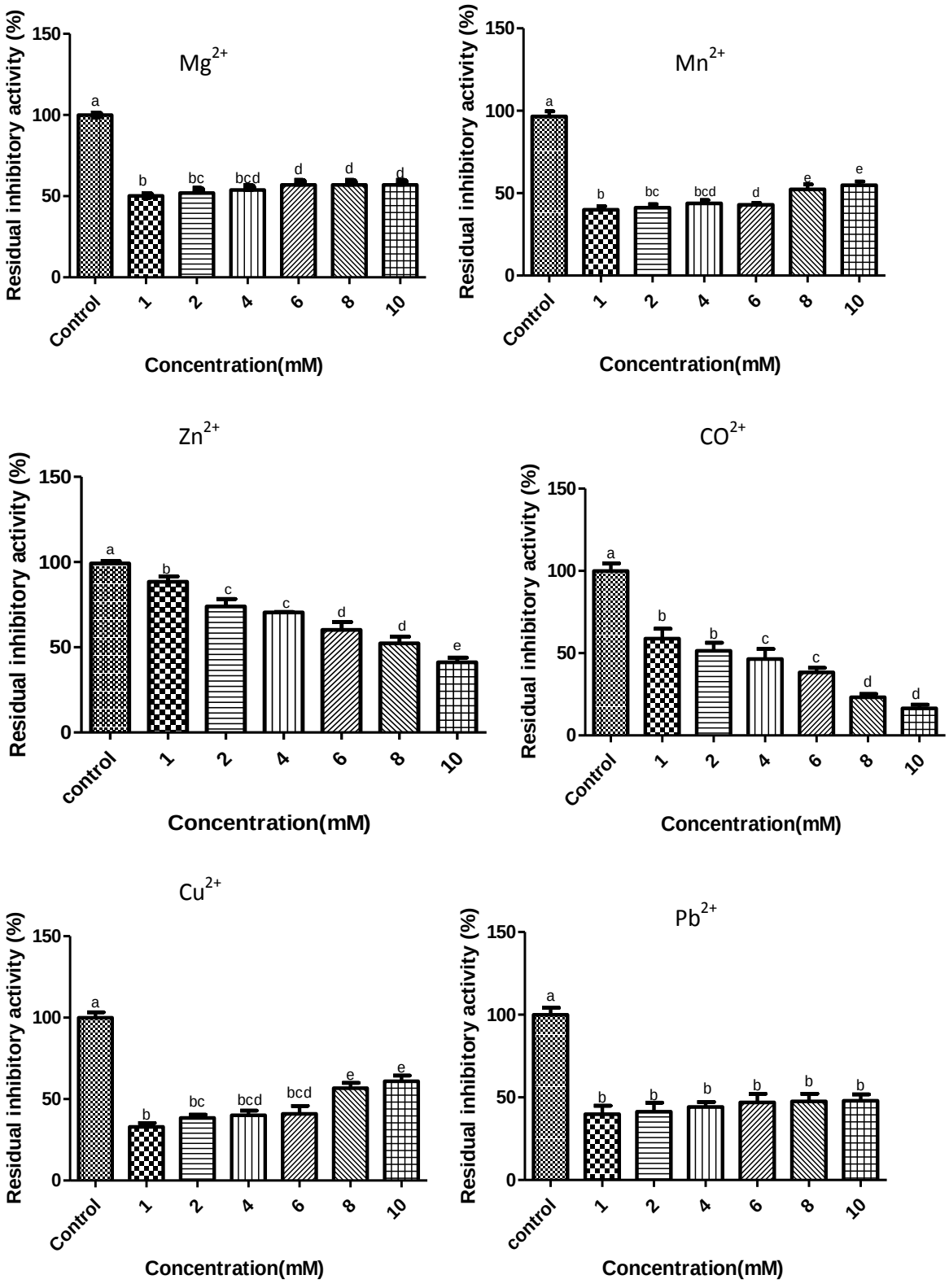

Figure 6. Effect of metal on CPI activity. The bars with the same superscripts are not statistically different ($P<0.05$).

cancer, atherosclerosis, and neurodegenerative diseases. However, further research is still required in determining its structure in other to elucidate its function and absolute specificity.

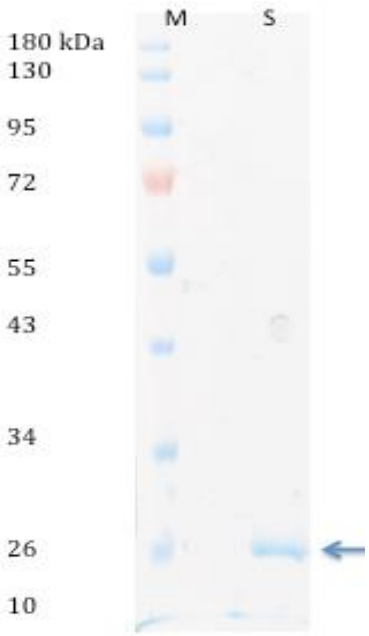

Figure 7. SDS-PAGE analysis of purified cysteine protease inhibitor. The gel pattern of the cysteine protease inhibitor fraction when subjected to SDS-PAGE yielded a single polypeptide band with a molecular weight of 20 kDa. M=marker; S=sample.

Conflict of interests

The authors did not declare any conflict of interest.

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Effects of sound waves on the enzyme activity of rice-*koji*

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Rice-*koji* is an important agent for the saccharification of fermented food production in Japan. In *koji*-making, temperature, humidity, light and oxygen are important environmental factors. However, there is no report of *koji*-making controlled by sound wave. In this study, focusing on the enzyme activity of rice-*koji*, we confirmed the relationship between enzyme activity and frequency of sound waves. Here, the enzyme activities of rice-*koji* cultured with no sound and sound waves at seven different frequencies (1.0, 2.5, 6.3, 8.0, 10.0, 12.5 and 16.0 kHz) were compared. Using two types of polished rice from different production areas, the activities were investigated. In this study, we investigated the enzyme activity of four types of rice-*koji* and found frequencies where the enzyme activity was significantly different to activity with no sound. In particular, the frequency of significant difference was investigated to confirm both rice. In the results, due to the irradiation frequency of 6.3 kHz, glucoamylase activity of both rice-*koji* decreased significantly ($P<0.01$) compared to the silent control. Further, due to the irradiation frequency of 1.0 kHz, acid protease activity confirmed a significant difference between the silent control and a frequency of 1.0 kHz ($P<0.01$). That is, as the activity of one of rice-*koji* increased, that of another rice-*koji* decreased. However, the activity of α-amylase and acid carboxypeptidase was not affected at any frequency. These results suggest that sound waves, particularly at frequencies of 1.0 and 6.3 kHz, have marked effects on the specific enzymatic activity of rice-*koji*.

Key words: Frequency of sound waves, sound wave, rice-*koji*, enzyme activity, *koji*-making.

INTRODUCTION

In the production of fermented food in the Orient region, rice-*koji*, which is prepared by culturing fungal conidia on cooked rice, is an important saccharifying agent. During the production of rice-*koji*, temperature, humidity and oxygen are key factors affecting quality (Murakami, 1988). Yanagiuchi et al. (1993) reported that the supply of carbon dioxide affected the fatty acid composition, color of *koji* and components of *koji* extract. On the other hand, Iemura et al. (1996) reported that *koji*-making sealed in *toko* period, that is, incubation period, was higher in the activities of α-amylase and glucoamylase, while, lower in the activities of acid protease and acid

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carboxypeptidase than *koji*-making not sealed up in *toko* period. Furthermore, it was recently confirmed that the germination of conidia is affected by light and the possibility of using light to control the production of rice-*koji* was demonstrated (Hatakeyama and Kitamoto, 2008). Yan et al. (2014) reported the optimum conditions for glucoamylase, α-amylase and acid protease of *Aspergillus oryzae koji*. Glucoamylase and α-amylase activity could reach maximum under the following conditions; inoculation temperature at 35°C, 0.25% inoculation quality, culture humidity at 75%, culture temperature at 37°C and 56 h culture time. On the other hand, acid protease could reach maximum when the culture temperature was 33°C. Zhang and Gao (2011) reported that the optimum condition for amino peptidase activity in wheat bran *koji* making were water ratio 100%, raw material cooking for 21 min and *koji* making for 44 h. However, there are no reports for utilization of sound waves during the rice-*koji* making process.

In Japan, sound waves have been used in various processes, including the aging of spirits, particularly *shochu*, cultivation of fruit and vegetables and even the breeding of cattle. For *shochu*, acoustic aging of black sugar-*shochu* has been reported. For vegetables, growth promotion has been reported. For livestock, relaxation effects have been reported. However, there are no reports as paper. It is only been empirically applied. The reason for this is that elucidation of scientific mechanism of sound effects is thought to be difficult. The mechanism is not understood although it was been suggested that sound waves could affect the rate of proton transfer in proteins (Barnes et al., 1985). Furthermore, it was reported that sound waves at a frequency of 2.0 kHz have been shown to affect the sugar composition of plants (Satoh and Tanisu, 2013). Based on these findings, we speculated that the production of rice-*koji* could be optimized using sound waves and thus attempted to determine the critical sound wave frequencies that would specifically impact the enzyme activity of rice-*koji*.

MATERIALS AND METHODS

Materials

Rice-*koji* was prepared using commercially purchased polished rice (*Koshihikari*) produced in Niigata and Miyagi Prefectures, Japan. Hereafter, the former was expressed as N-rice, the latter was expressed M-rice. Tane-*koji* (*Aspergillus oryzae*) was purchased from Kawachi Genichiro Shoten Co., Ltd., Kagoshima, Japan.

Rice-*koji* making

Rice-*koji* was prepared according to the method of Saigusa and Ohba (2007). Briefly polished rice (200 g) was soaked in water at 15°C for 20 min and the water was then drained over a 2 h period at 15°C. The rice was then steamed for 40 min in a pot-type steamer, removed, and allowed to stand until the temperature decreased to 40°C. Next, 0.2 g of Tane-*koji* spores was inoculated onto the steamed rice, which was then mixed to uniformly disperse the spores. Except for the steaming process, the cultivation procedure was performed under sterile conditions. After inoculation, the steamed rice was transferred to a Petri dish of 152 mm in diameter and compacted with a spoon. A piece of filter paper was inserted inside the cover, and the plate was incubated at 30°C for 20 h with no sound waves. A further 20 h to irradiate sound wave, rice-*koji* was moved to a tray which covered gauze and containing water in bottom. Incubation was performed in incubator built in speaker that is connected to the audio generator MINIRATOR MR2 (NIT Japan Co., Ltd, Tokyo, Japan) and instrument for amplification. The rice-*koji* was incubated in the presence of sound waves with frequencies ranging from 0 to 16.0 kHz. The sound power level of audio generator was keep -50 dBv. To keep moisture, making-*koji* was performed in plastic bag. The effects of sound wave frequency on the enzyme activity of glucoamylase, α-amylase, acid protease, and acid carboxypeptidase as main enzymes of rice-*koji* was then investigated. Rice -*koji* was prepared one each time. After extraction of enzymes from three places at random from both rice-*koji*, enzyme activity was measured.

Enzyme activity of rice-*koji*

Glucoamylase

Glucoamylase produces glucose from starch or oligosaccharides. Glucoamylase activity was measured with G2-β-PNP (4-nitrophenyl-β-maltoside) and PNPG (4-nitrophenyl-α-glucoside) as substrates using a commercially available kit (Kikkoman Co., Ltd., Chiba, Japan).

α-Amylase

α-Amylase produces oligosaccharides from starch. The α-amylase activity was measured with N3-G5-β-CNP (2-chloro-4-nitrophenyl 65-azido-65-deoxy-β-maltopentaoside) as a substrate using a commercially available kit (Kikkoman Co., Ltd.).

Acid carboxypeptidase

Acid carboxypeptidase produces amino acids from proteins and oligopeptides. The acid carboxypeptidase activity was measured with Cbz-Tyr-Ala (carbobenzoxy-L-tyrosine-L-alanine) as a substrate using a commercially available kit (Kikkoman Co., Ltd.).

Acid protease

Acid protease produces oligosaccharides from proteins. The acid protease activity was measured with casein as a substrate according to the method described by the National Tax Administration (1993).

Moisture content

The moisture content of steamed rice was measured using an FD-600 Infrared Moisture Determination Balance (Kyushu Kett Co. Ltd., Fukuoka, Japan).

RESULTS AND DISCUSSION

In this study, we aimed to prove that sound waves are

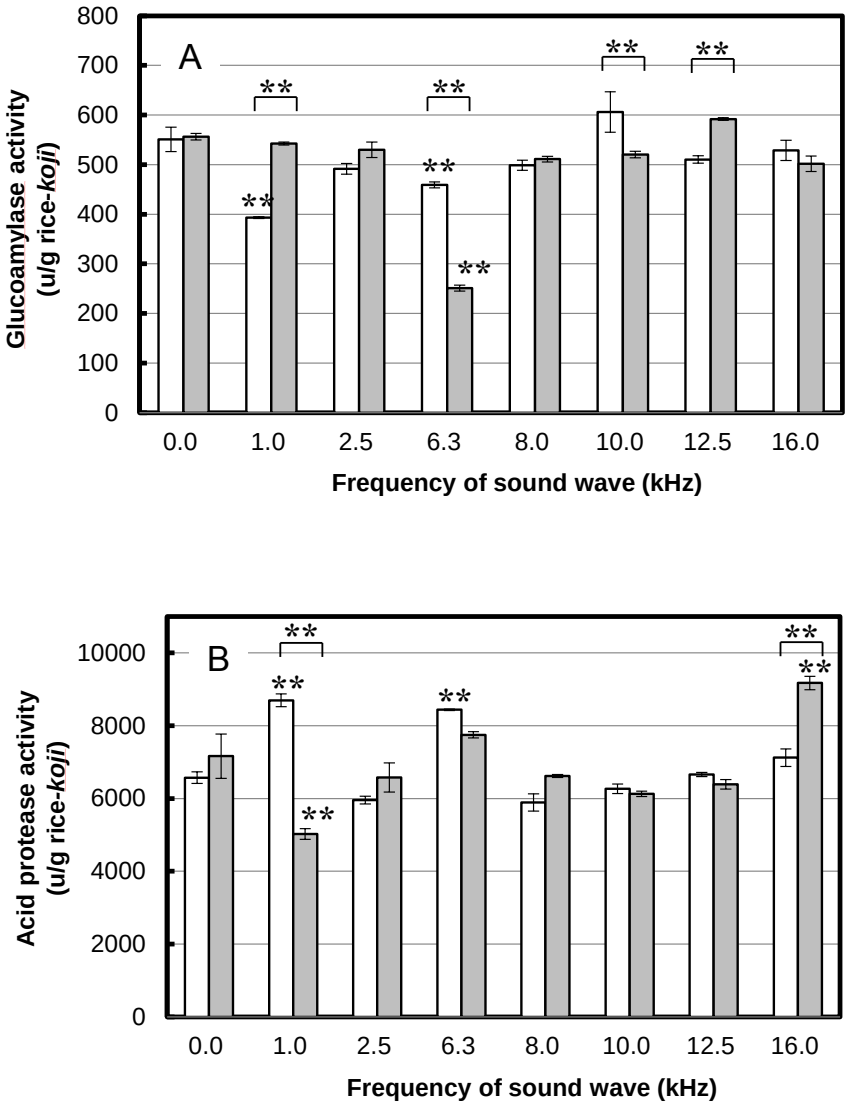

Figure 1. Effects of frequency of sound wave on enzyme activity of rice-*koji*. **A**. Glucoamylase activity. **B.** Acid protease activity. White bar showed N-rice, and black bar showed M-rice. Symbol (**) shows the significant difference (P<0.01). Especially, symbol (**) shown on the bar, indicated that there was significant difference between sound and no sound (0 kHz) condition (Scheffe's F test). Only the frequency of the indicated symbol (**) on the bar in both white and black, shows the frequency that affect the enzyme activity of rice-*koji*. Each value are presented as the mean ± S.D. (n=3).

one of the important environmental factors influencing the enzyme activities of rice-*koji*. To identify the critical frequencies affecting enzyme activity, rice-*koji* was produced in acoustic culture conditions using one of eight frequencies and the enzyme activities of the resulting rice-*koji* were compared.

For glucoamylase activity, significant difference was observed between no sound and a frequency of 6.3 kHz (Scheffe's F test). Also, glucoamylase activity of both rice-*koji* decreased significantly (P<0.01) (Figure 1A). On the other hand, for acid protease activity, significant difference was observed between no sound and a frequency of 1.0 kHz (Scheffe's F test). That is, as the activity of one of the rice-*koji* increased, another rice-*koji* decreased significantly (P<0.01) (Figure 1B). For α-amylase and acid carboxypeptidase activities, there were no frequencies at which significant differences were observed in both rice-*koji* (Scheffe's F test) (Figure 2A, B). In this experiment, three statistical analyses were carried as multiple comparison test. Results of significant different test in glucoamylase and acid protease activity were same results gotten for three methods, Scheffe's F

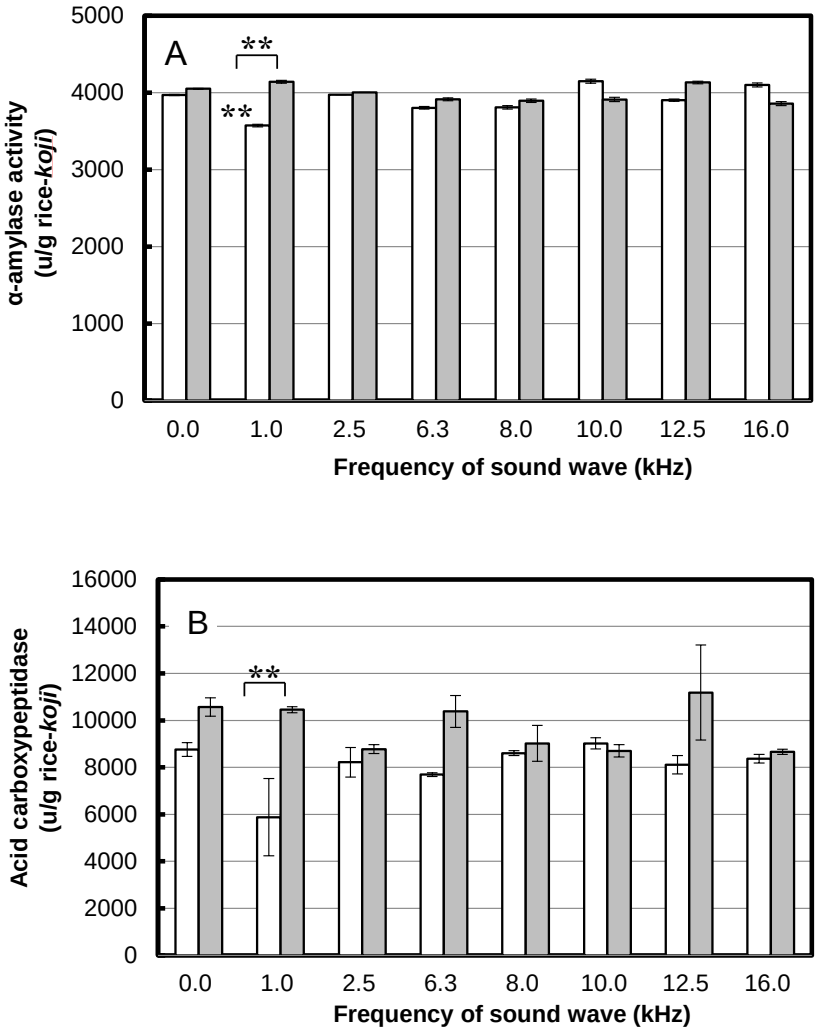

Figure 2. Effects of frequency of sound wave on enzyme activity of rice-*koji*. **A**. α-Amylase activity. **B.** Acid carboxypeptidase activity. White bar showed N-rice, and black bar showed M-rice. Symbol (**) showed the significant difference ($P<0.01$). Especially, symbol (**) shown on the bar, indicated that there was significant difference between sound and no sound (0 kHz) condition (Scheffe's F test). Only the frequency of the indicated symbol (**) on the bar in both white and black, shows the frequency that affect the enzyme activity of rice-*koji*. Each value are presented as the mean ± S.D. (n=3).

test, Tukey and Bonferroni/Dunn. Furthermore, from these analyses, we were unable to determine the cause and/or mechanism of the impact of frequency on rice-*koji* enzymes. To date, no studies have examined the effects of frequency on rice-*koji* enzymes. However, our findings do suggest that sound waves may be an important environmental factor influencing the rice-*koji* making process. In addition, we identified two sound wave frequencies (1.0 and 6.3 kHz) that might affect the enzyme activity of rice-*koji*.

We also observed that the rice-*koji* made from the two different sources responded differently to frequency of 1.0, 6.3 kHz. To determine the cause for this difference, the moisture content of rice-*koji* made from Niigata and Miyagi Prefectures was compared. Results show that the moisture content of cooked rice produced in Niigata Prefecture was 33.8%, while that of rice produced in Miyagi Prefecture was 40.7% (Table 1). Considering from the view point of vibration, it may be in close relationship between water and sound wave. In generally, the moisture content of steamed rice used in *sake* brewing is 27-32%. Furthermore, the more moisture content of steamed rice, enzyme activity decreases (Okazaki et al., 1979). However, in this study, it was confirmed that even when water content is high, enzyme activity increased significantly ($P<0.01$), by irradiating sound wave such as

Table 1. Water absorption and moisture content of rice used for rice-*koji*.

Rice	Water absorption rate (%) *1	Water absorption rate (%) *2	Moisture content (%) *3
N-rice	29.8	35.4	33.8
M-rice	35.0	40.2	40.7

*1 Absorption rate of water in raw rice. Calculation was performed as followed. [(weight of rice after drain – weight of rice before soak) / weight of rice before soak]×100. *2 Absorption rate of water in steamed rice. Calculation was performed as followed. [(weight of rice after steam – weight of rice before soak) / weight of rice before soak]×100. *3 Moisture content of rice after steam. Analysis method was described in the text. Both rice were produced in Sep. 2009 and purchased commercially in May 2010.

in glucoamylase (1.0, 12.5 kHz), acid protease (16.0 kHz), α-Amylase (1.0 kHz) and acid carboxypeptidase (1.0 kHz). Mishiro et al. (2000) reported that glucoamylase activity tends to increase according to the integration temperature of *ginjo koji*, that is starter for specially brewed Japanese sake. Okazaki et al. (1979) also reported the relationship between enzyme production and temperature. In that paper, they reported that α-Amylase and glucoamylase were produced well in optimum temperature of *koji* growth (37.5°C), while acid carboxypeptidase and acid protease were produced well under 35°C. In this study, in spite of constant temperature during *koji*-making, the difference of enzyme activity was observed by irradiation sound wave of various frequencies.

From these results, a possibility was suggested that a particular frequency might affect the enzyme production of rice-*koji*. In particular, with regard to 6.3 kHz, reduction of glucoamylase activity in the two types of rice-*koji* was also confirmed. Although the execution of repeat experiments is necessary, by the frequency of 6.3 kHz, a possibility of inhibition of the enzyme production or production of the active inhibitor is consider. Although the mechanism is not currently known about the effects of waves, overall impact of various factors, such as acoustic conditions, humidity, temperature and rice components (from the difference of water absorption rate), are considered.

As a result, enzyme activity does not depend at all on the high and low frequency. But, it was confirmed that each feature rice-*koji* was made with each frequency. From these results, the possibility was suggested that sound wave irradiation was one of the important environment condition for rice-*koji* making.

To further optimize the use of sound waves during the production of rice-*koji*, the underlying mechanism of frequency on enzyme production will need to be conclusively determined.

Conflict of interests

The authors did not declare any conflict of interest.

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