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EFFECT OF TEMPERATURE AND PARTICLE SIZE ON THE ALKALINE EXTRACTION OF PROTEIN FROM CHICKEN BONE WASTE

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Abstract

Chicken bone is a waste of chicken meat processing industry and restaurants that has not been used widely, even though it contains valuable organic compounds that are functionals, such as collagenous and non collagenous protein. This research was conducted to investigate the effect of temperature and particle size on the protein extraction from chicken bones using dilute sodium hydroxide solution. Controlled parameters in this study were the solvent in the form of sodium hydroxide solution, extraction time for 1 hour, pH 10.5, the ratio of chicken bone powder: solvent (1:4 w/v), and stirring speed 200 rpm. While the operating variables included the extraction temperature of 30^{o}C, 55^{o}C, and 80^{o}C, and particle size of 150 and 250 µm. Experiments were carried out by heating of 300 mL of sodium hydroxide solution with pH 10.5 in a three-necked flask equipped with Leibig condenser, thermometer, mechanical agitator and sampling device to reach the desired temperature (30^{o}C, 55^{o}C, and 80^{o}C). Then, a total of 75 g of chicken bone powders with desired particle size (150µm and 250µm) was introduced into the sodium hydroxide solution and the stirrer was operated at speed of 200rpm. At every 10 minutes interval, as much as 10 mL samples were withdrawn for total protein analysis using Lowry-Folin method. The experiment was terminated after 1 hour. The results show that both increase in temperature and particle size caused an increase in the amount of extracted protein. Highest concentration of protein extracted was achieved at 630.99 mg/L, when the extraction was carried out using 250µm bone particles and temperature 80^{o}C.

Keywords: *chicken bones, extraction, particle size, proteins, temperature*

Abstrak

Tulang ayam merupakan limbah yang dihasilkan oleh industri pengolahan daging ayam dan rumah makan yang belum dimanfaatkan secara luas, padahal tulang ayam mengandung senyawa organic fungsional yang tinggi nilainya, seperti protein kolagen dan non-kolagen. Penelitian ini ditujukan untuk mengkaji pengaruh suhu dan ukuran partikel terhadap ekstraksi protein tulang ayam dengan pelarut larutan soda api encer. Peubah yang dikendalikan dalam penelitian ini adalah pelarut yang berupa larutan soda api, waktu ekstraksi satu jam, pH 10,5, rasio berat tulang ayam terhadap volum pelarut 1:4 dan laju pengadukan 200 putaran per menit (ppm). Sementara itu, peubah yang diteliti adalah suhu ekstraksi (30^{o}C, 55^{o}C, dan 80^{o}C), dan ukuran partikel serbuk tulang ayam, yaitu 150 dan 250 µm. Percobaan dilakukan dengan memanaskan 300 mL larutan soda api dengan pH 10,5 dalam labu leher tiga yang dilengkapi dengan pendingin balik, termometer, pengaduk mekanik dan alat pengambil contoh hingga mencapai suhu yang diinginkan (30^{o}C, 55^{o}C, dan 80^{o}C). Kemudian, sebanyak 75 gram serbuk tulang ayam dimasukkan ke dalam larutan tersebut dan pengadukan dijalankan dengan laju 200 ppm. Sebanyak 10 mL contoh diambil setiap selang waktu 10 menit untuk dianalisis kadar protein totalnya dengan metode Lowry-Folin. Percobaan dihentikan setelah ekstraksi berlangsung selama satu jam. Hasil percobaan menunjukkan bahwa kenaikan suhu ekstraksi meningkatkan jumlah protein yang terekstrak. Selain itu, kenaikan ukuran partikel serbuk tulang ayam juga meningkatkan jumlah protein yang terekstrak. Kadar protein tertinggi dalam larutan contoh senilai 630,99 mg/L dicapai pada ekstraksi yang dijalankan pada suhu 80^{o}C terhadap serbuk tulang ayam yang berukuran 250µm.

Kata kunci: *tulang ayam, ekstraksi, ukuran partikel, protein, suhu*

INTRODUCTION

Indonesia has long been known as one of the most potential nations in animal agriculture. Poultry husbandry becomes a very popular activity as the main source of animal based protein for Indonesian. High demand in chicken meat has promoted steady rise of annual live stock production. According to Central Java Statistic Bureau, poultry production in this province reached 67,915,076 in the year of 2008 (www.disnak.jawatengah.go.id). Meat and eggs are produced from these animals, mostly for domestic consumption. However, the residual parts, such as skin, feather and bone are wasted. Skin, bone and feather contain large amounts of the structural proteins keratin and collagen. Keratin and collagen and associated hydrolysates have desirable characteristics for various industrial applications due to their functional properties (FP). Functional properties is the term given to a set of physicochemical characteristics of an individual protein, which determines its behaviour in aqueous media (swellability, solubility, gel formation, etc.), and in the heterophase systems of the type water-oil, oil-water (emulsifying capacity), or water-gas (capacity for foam formation), as well as in multicomponent mixtures. The functional properties of the protein are manifested as a function of its nature and the character of its interaction with other components of the given food system (Pavlova *et al*., 1989). Poultry based proteins are not hazardous to human health, are capable of water retention on the molecular scale, are essential biological nutrients and can serve as chromatographic carrier particles. Accordingly, they can be used in materials associated with or included in cosmetics, artificial organs, etc. and as such animal wastes in which these proteins are potentially valuable resources (Morimura *et al.*, 2002). These advantages have come out with an idea leading to extraction of chicken bone protein prior to enhance chicken bone economic value.

One of the simplest methods of protein extraction is by solubilisation of proteins using alkaline and acid hydrolysis. Since 1970, alkaline hydrolysis has been used widely for the improvement of the functional properties of fish protein concentrates (Moorjani and Vasantha, 1973). Such treatment usually increases the solubility and the emulsifying and foam-forming capacities of the protein. Treatment of myofibrillar proteins with alkalis and acids in order to cleave the peptide bonds induces profound irreversible denaturation changes and is accompanied by the appearance of a number of side reactions. There are some parameters that could affect the protein extraction efficiency, such as type of solvents, pH, temperature, particle size, time, feed/solvent ratio and agitation speed. The solvents used in the extraction have to easily dissolve the protein molecules, do not react with protein, and are non toxic. They can be acid or base, which is probably HCl and guanidine-HCl (Gotoh *et al*., 1995) or NaOH solution (Shahidi & Synowiecki, 1996). Protein extraction under acidic condition requires higher cost as it is commonly done at temperatures below room temperature. Refrigeration system is therefore inevitable, and considering economic point of view, alkaline extraction is then selected in this research. Shahidi & Synowiecki (1996) extracted protein from bone residues of harp seal *(Phoca groenlandica)* under alkaline condition. They reported that extraction of protein from harp seal bone residue at 80^{o}C obtained higher yield (13.07%) as compared to extraction at 20^{o}C, which only achieved 12.02% yield. From mass transfer point of view, the smaller the particle size of the starting material employed for solid-liquid extraction, the higher the extraction yield obtained. However, treatment of the starting materials and change them into fine particles requires high operational cost. Therefore, the economical particle size used for bone protein extraction is usually between 150-250 μm (Gerstenfeld *et al*., 1994). Acidic extraction of chicken bone protein needed about 1-7 days to achieve reasonable yield (Gotoh *et al*., 1995), while alkaline extraction of harp seal bone residues needed only 1 hour to achieve similar result (Shahidi and Synowiecki, 1996). High pH extractions and low pH precipitations have been used to isolate, concentrate, and structure proteins from vegetable sources (Fletcher and Ahmed, 1977). Similar high pH extractions (pH of approximately 10.5) followed by acid precipitation have also been conducted on poultry and red meat residues and by-products to concentrate the protein fractions while still maintaining a fibrous structure (McCurdy *et al*., 1986). High pH extractions are often accompanied by the degradation of alkali-labile aminoacids, the partial racemisation of aminoacids, and the β-elimination process with participation of hydroxy- and mercapto-aminoacids leading to the formation of dehydroalanine, which readily interacts with lysine, cysteine, ornithine, and ammonia (Chung *et al*., 1986). The products of these reactions are lysinoalanine, lanthioline, ornithinoalanine, and β-aminoalanine, respectively. This diminishes the food value of the protein by depleting it in essential aminoacids, by the partial loss of assimilability as a result of the formation of intermolecular crosslinks, and by the accumulation of antinutrient substances. The question of the toxicity or harmlessness to man of dehydroalanine derivatives still remains open (Tolstoguzov, 1987). Comparatively, recently it has been demonstrated experimentally (on skeletal muscles in fish) that lysinoalanine is formed only under very severe alkaline conditions at pH 12 and above and at temperatures not below 90°C (Miller *et al*., 1983). Thus the possibility of using relatively mild hydrolysis for food proteins has not been rejected at the present time (Richardson and Kester, 1984). Extractions conducted at pH 10.5 or less are not associated with producing potentially dangerous compounds, such as lysino-alanine (Ozimek *et al.*, 1986). Unfortunately, effective degradation or hydrolysis of materials

composed of keratin or collagen is difficult due to their stable hard protein structure, thus industrial utilization in materials for food, cosmetics, artificial organs etc. of these proteins have been little developed (Morimura *et al*., 2002). To ensure that effective molecular interactions between solvent and bone particle take place, the ratio of bone particle to solvent is at least 1: 4 (w/v) (Shahidi and Synowiecki, 1996). No literature reporting on agitation speed in chicken bone protein extraction. Fortunately, Board *et al*. (2008) reported that highest yield was obtained in cow bones protein extraction using 200 rpm agitation speed.

The recent excess of broiler leg quarters and dark meat productions, and the resulting huge amount of bones waste suggests the possibility of enhancing chicken bones utilization by extracting and producing a highly functional, low fat, light-colour protein extract (Betti and Fletcher, 2005). The purpose of this project was to examine the effect of temperature, ranging from 30, 55 and 80^{o}C, and particle size, ranging from 150 µm and 250 µm on the extraction of soluble protein from broiler bones. This project focused on dry weight yield because this would be a dominant issue regarding economical use of such an extract.

MATERIAL AND METHODS

Raw Materials and Chemical Reagents

The chicken bones waste used in this study were collected from McDonald Jl. Setia Budi, Semarang. Proximate analysis using the method suggested by Aerssens *et al.* (1998) reported that the dry bone powder contains 7% (w/w) of collagenous protein. Sodium hydroxide of analytical grade (99% purity), n-Hexane of analytical grade (99.5%), and HCl of reagent grade (37%) were purchased from Merck, Germany. Laboratory grade water was supplied by reverse osmosis (RO) unit in the Department of Chemical Engineering, Faculty of Engineering, Diponegoro University, Semarang.

Pretreatment

For removal of fat from bone, 60 g of dried sample was placed in a glass roller bottle (3 L capacity) and 400 mL of hexane was added to the bottle, which was then agitated on a roller-culture apparatus (Wheaton Instruments, NJ, USA) at a speed of 200 rpm at room temperature for 12 hours. The material in the roller-bottle was filtered through no. 5A filter paper (ADVANTEC, Tokyo) and dried for 24 hours under the conditions of 60°C and reduced pressure at -76 mmHg.

Removal of inorganic compounds from bone was carried out by soaking it in a 0.6 N HCl solution at room temperature for 24 hours. The concentration of bone in the solution was 10% (w/v). The de-ashed bone was filtered through no. 5A filter paper and washed three times with tap water (same volume as used for the 0.6 N HCl solution). The procedure for removal of inorganic compounds was performed twice, in which case the soaking and washing series described above was repeated. The sample was then dried for 72 hours under the conditions of 60°C and reduced pressure at -76 mmHg. The dried bone was ground to powder using a mill (Personal mill SCM-40A, Shibata Scientific Technology Ltd., Tokyo) and sieved to obtain average bone particle sizes of 150 µm and 250 µm.

Extraction of Bone Protein

The alkaline solution was prepared by diluting a NaOH solution with water to pH 10.5. Three hundreds millilitres of the solution was then introduced into a 500 mL capacity of three necked flask, equipped with Leibig condenser, thermometer, mechanical agitator and a sampling device (Figure 1).

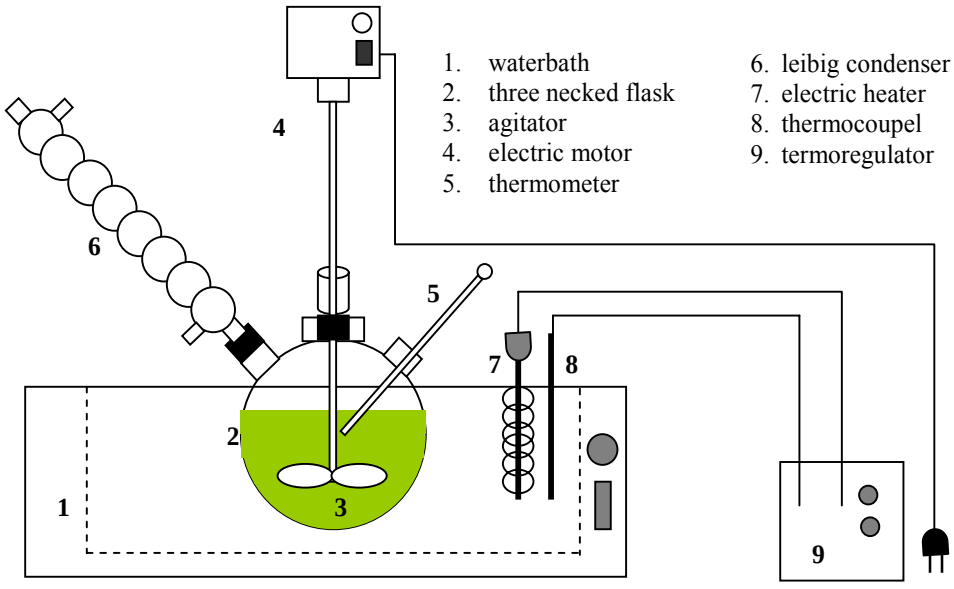

Figure 1. Experimental Set-up

The system was then heated to a desired temperature by immersing the flask in a water bath heater. Once the desired temperature was achieved, 75 grams of chicken bone particles was then added into the warm NaOH solution and the agitation was started. Extraction was done at three different temperatures of 35°C, 55°C and 80°C at a rotation speed of 200 rpm. Liquid samples were withdrawn at every 10 minutes interval, and the extraction was terminated after 1 hour. After extraction, the sample was filtered with a double layer of gauze and the filtrate was centrifuged at 8000×*g* for 10 min. The supernatant was then lyophilized and the resulting extract was used for further investigations. For determination of the protein content, 60 mg of dried sample and 5 mL of 1 N NaOH solution were added to a test tube and mixed well. The tube with sample was then heated in boiling water for 10 min and cooled to room temperature. The extracted solution under alkaline conditions was filtered and made up to 100 mL. The protein content was then determined by the Lowry-Folin method (Lowry *et al*., 1951). Collagen peptide reagent (Wako Pure Chemical Industries Ltd., Osaka) was used as the standard.

RESULTS AND DISCUSSIONS

The results of obtained from the extraction of protein from chicken bones are illustrated in Figure 2 and 3.

Effect of Temperature

Figure 2 shows that as extraction temperature increases, the highest protein content in the sample extracted in 1 hr also increases. This indicates that more protein was extracted at higher temperature. The same trend was found that as the extraction time prolonged, the amount of extracted protein also significantly enhanced for all temperatures under investigation. Similar results were reported on the extraction of protein from fish bone waste (Batista, 1999) and harp seal bone residue (Shahidi and Synowiecki, 1996). Figure 2 also suggests that extraction at 80°C was the fastest, and extraction for 30 minutes has already achieved the maximum content of extractable protein. Extraction of chicken bone protein at 55°C for 60 minutes yielded a comparable result as extraction at 80°C for 30 minutes. The figure also indicated that extraction was slowest at 30°C. Thermal conductivity and specific heat capacity of chicken bones are 0.265 W/mK and 2.021 kJ/kg.K, respectively (Siripon *et al*., 2007). These two thermal properties affected the heating mechanism of chicken bone protein in sodium hydroxide solution. In the beginning of the extraction, the heat supplied by water bath was mainly used to increase bone temperature to the desired temperature rather than for the extraction itself. Once the desired temperature was achieved, the heat was then primarily used for extraction. This phenomenon is fairly proven in this work for extraction at 55°C and 80 °C, where the extraction rate was very slow in the beginning (0-20 minutes) and followed by fast extraction (20-60 minutes). Rigorous discussion for the effect of temperature on the chicken bone extraction can be done based on data reported in Table 1.

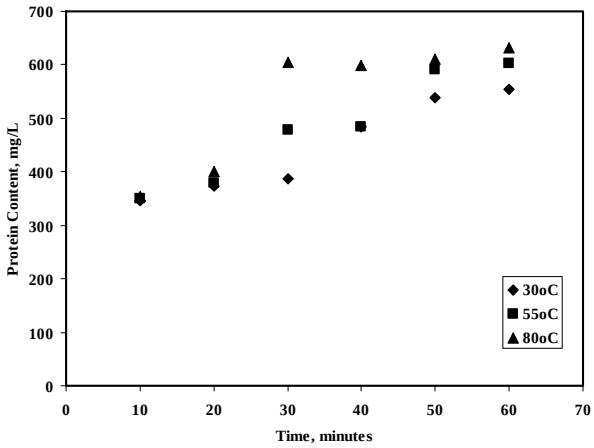

Figure 2. Effect of Temperatures on the Extracted Protein in the Alkaline Extraction using 250 μm Particle Size

Table 1 show that the mass diffusivity of chicken bone protein in dilute sodium hydroxide solution increases with the increases in temperature. Increase in mass diffusivity will speed up the mass transfer rate of protein from bone particles to sodium hydroxide solution. When temperature increased, both protein and solvent molecules moved fast, mass transfer rate of interface between solid and liquid developed.

Table 1. Parameters used for theoretical analysis in effect of temperature on chicken bone protein extraction

Temperature (°C)	Diffusivity Coefficient (cm^2/s)[a]	ΔV (volumetric expansion) (cm^3)[b]	Reaction rate constant (1/min)[c]	Maximum Protein Content (mg/L)
30	2.67×10^{-9}	3.23×10^{-9}	6.33×10^{-4}	447.33
55	2.89×10^{-9}	1.94×10^{-8}	6.86×10^{-4}	480.88
80	3.17×10^{-9}	3.55×10^{-8}	7.57×10^{-4}	533.20

[a] predicted using Wilke-Chang equation (Wilke and Chang, 1955)
[b] predicted using data from (Lang, 1969) and freeware available at www.engineeringtoolbox.com
[c] calculated based on first order reaction

Therefore, increasing temperature could promote mass transfer and solubility, reduce viscosity of solution and thus increase extraction rate (Zhang *et al*., 2009). However, high temperature could cause the reduction of protein activity and thermal denaturation of protein.

At higher temperature (80^{o}C), chicken bone particle matrices are more porous compared to that at lower temperatures (30^{o}C and 55^{o}C) as indicated by their volume expansion values listed in Table 1. The volume expansion was calculated based on volumetric expansion coefficient for chicken bone particles (79×10^{-6} K^{-1}) reported by Lang (1969). As a result, the soluble chicken bone protein is easier to diffuse from bone matrix to the sodium hydroxide solution at higher temperature, and finally more protein was extracted.

Assuming that the number of sodium hydroxide molecules was in excess, the kinetic of first order reaction for alkaline hydrolysis of chicken bone protein was investigated. The reaction rate constants obtained are also reported in Table 1. The rate constant increases with temperature because when temperature goes up, the number of effective molecular collisions to initiate reaction increases. This phenomenon supports the fact that the hydrolysis reaction was faster at higher temperature than that at lower temperatures.

Effect of Particle Size

Figure 3 shows that extraction of chicken bone protein was more pronounced at extraction using chicken bone particles of 250 μm than 150 μm. According to Senapati *et al.* (2009), a decrease in particle size (particularly when the particle size distribution is narrow) results in an increase in slurry viscosity, especially at low shear rates. As the slurry viscosity increases, with a consequent decrease in diffusivity, then the mass transfer coefficient also decreases (Mubarak *et al*., 2004). Therefore, more protein was extracted from chicken bone particles of 250 μm than 150 μm. This result is contradictive to the effect of particle size on the extraction of protein from soybean reported in the literatures. The amount of protein extracted from pea flour at pH 2 dropped from 73.6% to a mere 37.4% when the mean particle size of flour increased from 200 to 1000 μm (Le Gall *et al*., 2005). Russin *et al*. (2007) reported that the protein recovery improved from 40% to 52% without having any detrimental impact on the purity of the final soy protein isolate by decreasing the average particle size of starting raw material (defatted soy flour) from 223 to 90 μm. Further theoretical analyses were then done based on the data listed in Table 2.

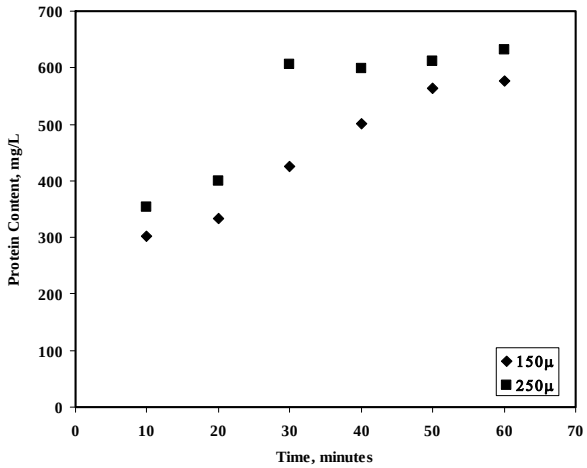

Figure 3. Effect of Particle Sizes on the Extracted Protein in the Alkaline Extraction at 80^{o}C

With turbulent flow in mechanically agitated tanks, there are frequent erratic changes in the directions of local fluid movement. There are also instabilities in the gross circulatory flow patterns, particularly in the transition zone between downflow core and upflow annular regions. As a result, there may be very little wake development associated with either suspended or fixed solute particles in these systems (Miller, 1971). The turbulence microscale as a measure of the size of eddies at the small eddy equilibrium end of the turbulence energy spectrum for nonviscous agitated systems values fall typically in the range between 10 to 150 microns. Boundary layer development seems unimportant as a mass transfer consideration when the solute particles approach the turbulence microscale dimension. In reviewing mass transfer data obtained in the small particle flow regime by Harriott (1962), the author determined that radial diffusion becomes dominant as the mass transfer mechanism at particle sizes of 205 microns and particles Reynolds number as low as within 0.1 to 10. As the chicken bone particle sizes were very small, but highly porous and brittle, the effect of intraparticle diffusion in considerably negligible. This suggests that extraparticle diffusion will be the mass transfer limiting step. As the particle size increases, the Reynolds number of the particles also increases.

The increase in Reynolds number also shows the increase of system turbulence (Perry, 1985). The more turbulent the flow of fluid in a system, the more severe mass transfer of protein between chicken bone protein to sodium hydroxide solution.

Tabel 2. Parameters used for theoretical analysis in effect of particle size on chicken bone protein extraction

Particle size (μm)	Reynolds numbers	Sherwood numbers	Maximum Protein Content (mg/L)
150	9.97×10^{-5}	9.99	450.56
250	3.08×10^{-4}	15.55	533.20

Investigation was done further by calculating bone particle's Sherwood number as suggested by Jadhav and Pangarkar (1990). The calculated Sherwood number reported in Table 2 shows that the bigger particles (250 μm) have higher Sherwood number suggesting higher mass transfer coefficient or mass transfer rate. Therefore, more protein was extracted when chicken bone protein was extracted using particle size of 250 μm. Similar trend was reported by Furusawa and Smith (1973) on their study on mass transfer between solid particle and liquid as slurry in agitated vessel.

Laboratory observations also revealed that at the same agitation speed of 200 rpm, bone particles of 250 μm mixed better with sodium hydroxide solution as compared to bone particles of 150 μm in size. The 150 μm bone particles tended to float and not dispersed well. This fact reduced the effective molecular interaction between bone particle and sodium hydroxide solution, and finally the amount of protein extracted.

CONCLUSIONS

The results of this research suggested that the following conclusions can be drawn. An increase in extraction temperatures from 30^{o}C to 80^{o}C increases the amount of extracted soluble protein from chicken bone waste. The amount of extracted soluble protein from chicken bone waste also increases with the increase in particle size from 150 μm to 250 μm. Highest concentration of protein extracted was achieved at 630.99 mg/L, when the extraction was carried out using 250μm bone particles and temperature 80^{o}C.

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