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ORIGINAL ARTICLE

Effect of ensiling and organic solvents treatment on proteolytic enzymes of layer chicken intestine

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Abstract Effect of ensiling and organic solvents on the protein extractability and on the activities of the proteolytic enzymes of layer chicken intestine was evaluated. The protein content of water extract of layer chicken intestine was 2.45 mg/ml. Highest proteolytic activity (26.3 units) was observed at pH 10.6 and lowest at pH 6.8 (7.2 units). Higher acidic proteolytic activities were observed at pH 2.4, pH 2.8 and at pH 5. Acid ensiling resulted in 65.6% reduction in acidic protease activity compared to 57.4% reduction by fermentation ensiling. Neutral and alkaline protease activities were also reduced up to a maximum of 41.8% and 46.5%, respectively. A ratio of 1:1.5 of cold acetone to intestine homogenate was found best for enhancing the neutral and alkaline protease activity. The study revealed that layer chicken intestine is a rich source of proteases especially of neutral and alkaline proteases, which could be harvested for commercial purposes and both acid and fermentation ensiling of layer chicken intestine resulted in reduction of protein extractability and enzyme activity. Treatment with acetone almost doubled the activities of neutral and alkaline proteases compared to initial values in water extract of fresh intestines.

Keywords Layer chicken intestine · Proteases · Ensiling

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Introduction

India has 1000 million broiler and 140 million layer population and every year about 6.8×10^7 tonnes of poultry meat are processed globally (FAO 2005). Huge quantities of edible and non-edible by-products such as intestine, feet, feathers and head are generated every year (Ockerman and Hansen 2000). Poultry intestine accounting for more than 20% of processing wastes is a potential source of protein including proteases (Raju et al. 1997, Ockerman and Hansen 2000).

Most of the non-edible by-products of poultry processing are discarded, resulting in environmental problems as well as loss of nutrients and valuable biomolecules. Generally, these wastes are converted in to fertilizers or animal feeds. Wastes such as broiler chicken intestines and fish viscera are ensiled for feeding animals (Raa and Gildberg 1982, Mahendrakar et al. 1995, Rathina Raj et al. 1996). By-products, mainly intestines are rich in proteases, lipases and collagen. Recovering and utilizing these nutrients and biomolecules is an important aspect in waste management in meat processing industries.

Animal tissues are rich in peptidases which play an important role in determining the quality attributes and storage characteristics of meat and fishery products (Kirstensen and Purslow 2000, Jamadar and Harikumar 2005). Proteases from animal tissues are generally classified as acidic (pH 4.0), neutral (pH 7.0) and alkaline (pH 8.0) (Sahidi and Kamil 2001). Many reports are available on the recovery, characterization and utilization of proteases, lipases and pigments from fishery industry wastes (Gildberg 2004, El-Beltagy et al. 2004, Sachindra et al. 2005, Bhaskar et al. 2007). Few reports on the recovery of proteinaeous substances from broiler chicken head (Surowka and Fik 1992, 1994) and intestine (Jamadar and Harikumar 2005) are also available. Intestinal proteases of chicken are comparable with those of liver and spleen (Jamadar and Harikumar 2005). No reports on the effect of ensiling and

organic solvents on the proteases activity of layer chicken intestine are available. Against this background this study was carried out.

Material and methods

Layer chicken (*Gallus gallus*) intestine was procured from local market and transported to the laboratory under chilled condition. All the chemicals used were of analytical grade.

Preparation of crude enzyme extract: Minced chicken intestine (25 g) was homogenized with 250 ml of ice cold distilled water using Polytron homogenizer (Model PT 3100, Kinematica AG, Littau, Switzerland) at 2000 rpm for 5 min. The homogenate was centrifuged at 5000 rpm for 10 min at 4°C, and the supernatant collected. The supernatant was filtered through Whatman Nr 41 filter paper and made up to 250 ml, which is referred to as crude enzyme extract and was used for the assay of protein and enzyme activity.

Determination of extractable protein: The protein content in the extracts was determined by the method of Lowry et al. (1951) and calculated based on bovine serum albumen (BSA) standard curve; 50 µl sample was used for protein assay.

Effect of pH on enzyme activity: In order to know the pH optima of enzymes, the activities of crude enzymes in the extract were determined at pH range of 2.4 – 10.6 using different buffers as per the method of Chong et al. (2002). HCl-glycine, CH_3COOH-CH_3COONa, Na_2HPO_4-NaH_2PO_4, and NaOH-glycine buffers (at a final concentration of 0.1 M) were used in the pH ranges of 2.4–3.6, 4.0–5.6, 6.0–8.0, 8.6–10.6, respectively. For pH range of 2.4–6.8, 1% haemoglobin and for pH range of 7.0 and 10.6, 1% casein were used as substrates. The assay mixture contained 1.25 ml buffer, 0.5 ml substrate and 0.25 ml enzyme extract. The assay mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 3 ml of 5% trichloroacetic acid (TCA). For control, TCA was added before adding the enzyme extract. The TCA extract was obtained by filtering the assay mixture using Whatman Nr 1 filter paper. The TCA soluble peptides in the TCA extract were determined by the method of Lowry et al. (1951) and represented as tyrosine equivalents by referring to tyrosine standard curve. The specific activity of enzyme was expressed as µg tyrosine released per mg protein per min.

Effect of ensilaging on enzyme activity: For preparation of acid ensilage, formic acid and propionic acid mixture (1: 1) was added to homogenized visceral mass and thoroughly mixed till pH reached 4.0. For preparation of fermented silage, homogenized viscera was mixed with 10% glucose and 2% NaCl. Addition of NaCl arrests gas producing microorganisms in the silage (Mahendrakar et al. 1995). The ensilages were stored for 5 days at room temperature (27 ± 2ºC) under micro-aerophilic condition as described by Mahendrakar et al. (1995). Enzyme from silage was extracted using distilled water and protein content and enzyme activity were determined as explained earlier.

Effect of organic solvents treatment on enzyme activity: Cold organic solvents (acetone and ethanol) were added to homogenised visceral mass in ratios of 1: 1, 1:1.5 and 1:2 (visceral mass: solvent; w/v) and further homogenized for 5 min at 2000 rpm. The homogenized mixture was filtered using Whatman Nr 1 filter paper. The residue was homogenized with organic solvent, filtered and treatment with organic solvent was repeated again. The residue was then dried under vacuum at 40°C and dried residue was stored in airtight glass vials at 5°C till analysis. For determination of enzyme activity in solvent treated powder, 1 g of powder was extracted with 50 ml cold distilled water to obtain the crude enzyme extract. The protein content and the enzyme activity were determined as mentioned earlier.

Statistical analysis: The experiments were repeated 3 times. The significant differences between treatments were analysed using ANOVA and mean separation was accomplished by Duncan's multiple range test using STATISTICA (Statsoft 1999).

Results and discussion

Effect of pH on enzyme activity: The protein content of the water extract of layer chicken intestine was 2.45 ± 0.08 mg/ml. In the acidic range studied, the activity of the enzyme reduced from 17.8 (pH 2.4) to 9.0 units (pH 6.0) (Fig. 1). In fact, at pH 6.8, the activity of the enzyme was least among all the pH levels studied. Further a sharp increase in the activity at pH 7 was observed followed by slight decrease up to pH 8.0. From pH 8 onwards, the activity steadily increased reaching a maximum of 26.3 units at pH 10.6. Similar results were reported in the literature on proteolytic enzymes with optimum pH in the acidic range of pH 2.4–5.0 in fish viscera (Chong et al. 2002) and in alfalfa weevil mid gut (Wilhite et al. 2000) and in the alkaline range of pH 7–10 (Giménez et al. 2000, Wang et al. 2002). The results indicated that the protease extracted from layer chicken intestine has highest activity in the alkaline pH range. Further, the pH profile for specific activity showed many peaks at pH 2.4, 2.8, 5, 7 and from pH 8.6 onwards there was gradual increase in the activity. Similar results were observed in trout heads (Michail et al. 2006).

Effect of ensilaging on enzyme activity: The extractable protein decreased during acid ensiling (Fig. 2). The reduction in buffer extractable protein during acid ensilaging was due to precipitation of proteins at reduced pH conditions (Bhaskar et al. 2007). In the case of fermentation ensilaging, significant reduction in extractable protein from the initial value of 2.4 ± 0.02 to 1.9 ± 0.03 mg/ml on 5th day of ensiling was observed. Significant ($p \leq 0.01$) difference was observed in protein extractability between acid and fermentation ensiling during the storage of silage up to 5 days. The reduction in the extractable protein during fermentation ensiling may be due to the interference of sugars in extractability of proteins.

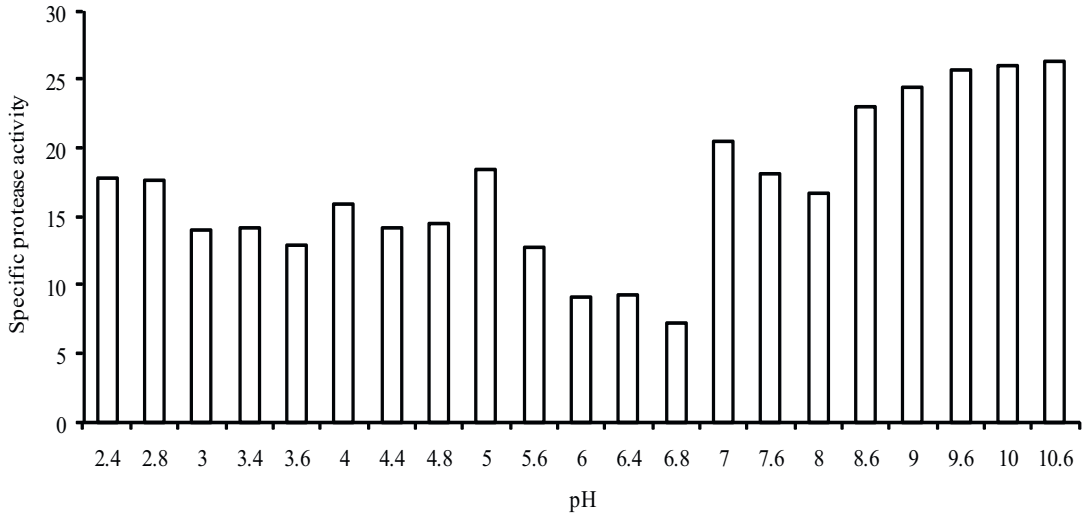

Fig. 1 Specific activity (μg tyrosine released/mg protein/min) of proteases in the crude extract of layer chicken intestine at different pH (n=3)

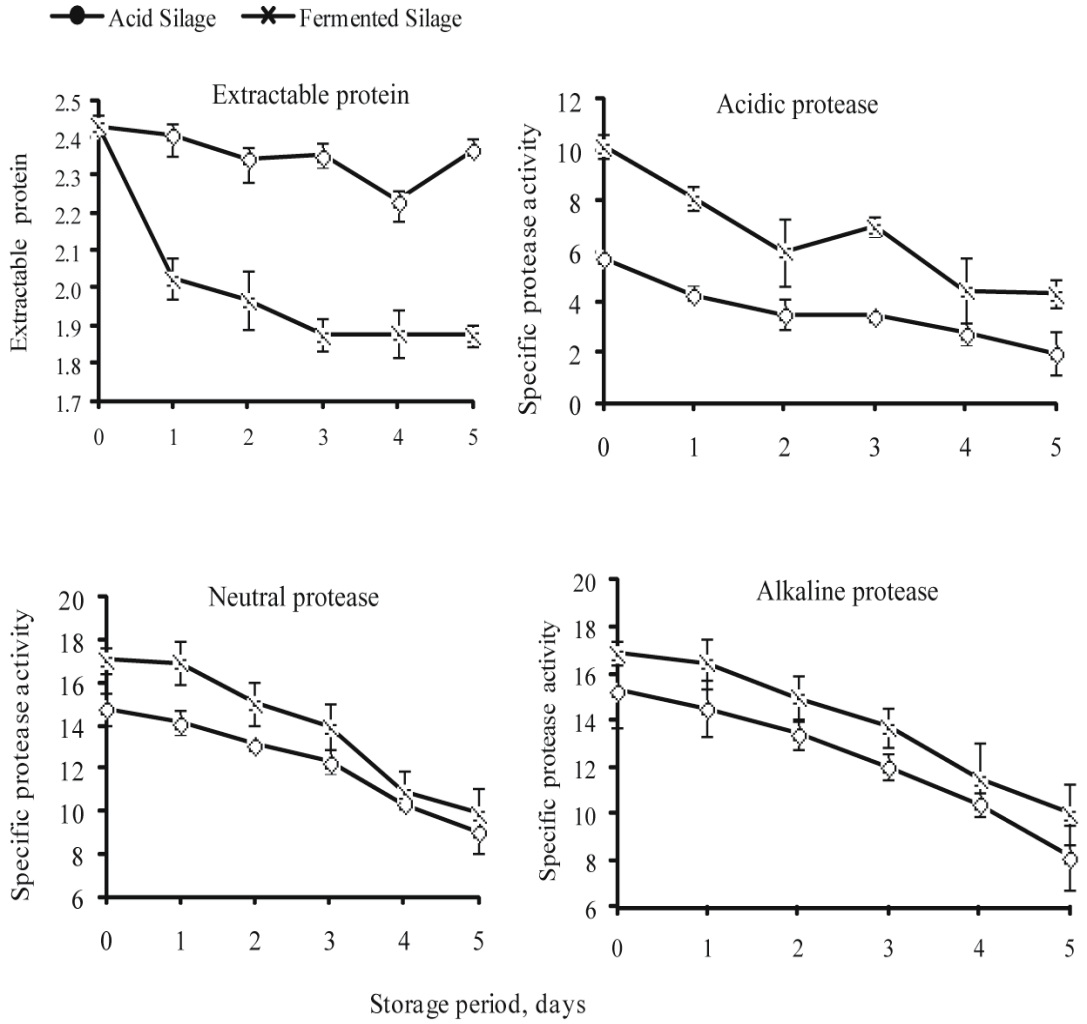

Fig. 2 Extractability of proteins (mg/ml) and spcific activity (μg tyrosine released /mg protein/min) of proteases during ensiling of layer chicken intestines (n=3)

The reduction in the acidic protease activity in the acid silage was 25.1 % which reached to 65.6 % on the fifth day when compared to the initial value (Fig. 2). Similar was the change in fermentation ensiling. Acidic protease activity was affected significantly ($p \leq 0.01$) in both acid and fermentation ensiling but not the neutral and alkaline proteases. In acid ensiling, the neutral protease activity reduced from the initial 14.8 ± 0.58 units to 12.3 ± 0.57 units on third day to 9.0 ± 1.04 units on fifth day (Fig. 2). In other words, a reduction of 38.64 % in the activity of acidic protease in acid ensilaging occurred. During fermentation ensiling there was also a reduction in the activity of neutral protease up to the extent of 41.8% (from 17.1 ± 0.61 to 9.9 ± 1.21 units). In the case of alkaline protease activity also there was a reduction on 5th day (Fig. 2).

Acid ensiling and fermentation ensiling are commonly used for preserving the animal by-products (Gildberg 2004, Mahendrakar et al. 1995). Acidic protease activity is stable in ensiled fish viscera (Reece 1988). However, Bhaskar et al. (2007) reported complete loss of protease activity in acid ensiled fresh water fish viscera indicating that acid ensiling is not a suitable method for stabilization of proteases in fresh water fish viscera. No reports are available on the effect of ensiling of layer chicken intestine on the proteases activity. The study revealed that acidic proteases from layer chicken intestine were more susceptible to ensiling than alkaline and neutral proteases.

Effect of organic solvent treatment on enzyme activity: Treatment with organic solvents removes most of the fat and moisture (Thomson et al. 1973) resulting in enzyme rich 'protein powder'. No significant difference was found in protein extractability of acetone and ethanol treatments (Fig. 3). Water extract of protein powder obtained from acetone treated intestine homogenate and solvent ratio of 1:2 had protein content of 6.2 mg/ml (Fig. 3.1c) when compared to 4.6 and 5.8 mg/ml in 1:1 (Fig. 3.1a) and 1: 1.5 (Fig 3.1b) ratios, respectively. In ethanol treated samples also 1:2 treatment groups had highest protein extractability (5.7 mg/ml) (Fig. 3.2c), and marginally lower protein contents were observed in 1:1 (Fig. 3.2a) and in 1:1.5 (Fig. 3.2b) solvent treatments. The activity of acidic protease in fresh intestine extract was 9.5 ± 0.42 units whereas in ethanol treated samples it ranged from 2.5 (Fig. 3.2a) to 6.2 (Fig. 3.2c) units and these values were significantly ($p \leq 0.05$) lower than the corresponding acetone treated samples.

Neutral protease activity was 16.1 ± 0.66 units in the fresh intestine and significantly ($p \leq 0.05$) increased to the maximum of 31.58 units in intestine homogenate: acetone ratio of 1:1.5 treatments (Fig. 3). Ethanol treatment significantly ($p \leq 0.05$) reduced the neutral protease activity. It ranged from 7.99 to 13. 96 units, much lower than 16.12 units as observed in untreated. The percent reduction ranged from 14.4 in 1:1 to 49.6 in 1:2 treatments.

Alkaline protease activity increased to the maximum of 29.7 units in tissue homogenate: acetone (1:1.5) treatment as against 16.5 units in fresh extract, an increase of 80.2%. Much lower values of alkaline protease activity of 10.1 units (homogenate: ethanol) 1:1 treatment to 14.6 units in 1:2 treatment. The percent reduction due to ethanol treatment ranged from 10.4 (in 1:2) to 49.6 (1:1). Layer chicken intestine homogenate and cold acetone in the ratio of 1:1.5 (wt/vol) was found to be best suited for protease extraction. Michail et al. (2006), Popova and Pishtiyski (2001), Maehashi et al. (2002) and Olivas-Burrola et al. (2001) have also reported that cold acetone is a better purification agent.

Conclusion

Layer chicken intestine is a good source of proteolytic enzymes, mainly neutral and alkaline proteases. Both acid- and fermentation ensiling reduced protein extractability

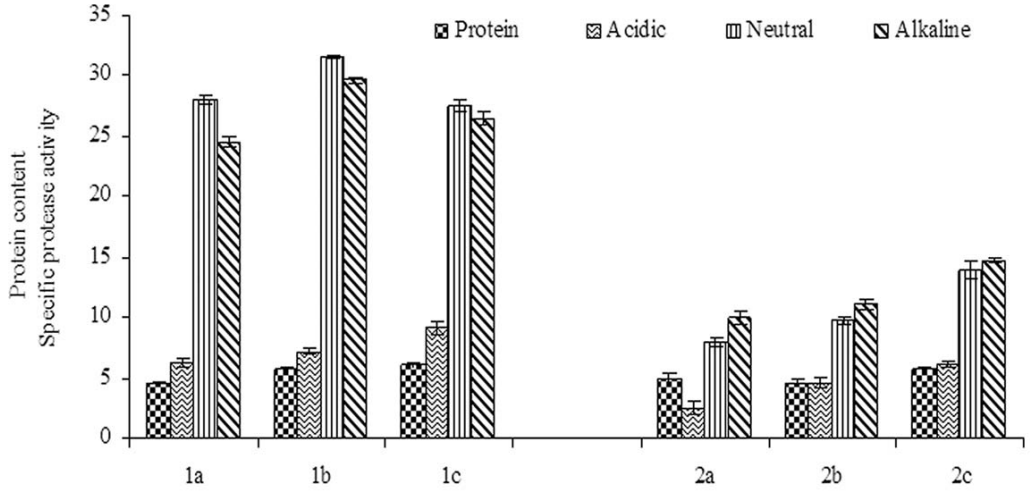

Fig. 3 Protein content (mg/ml) and spcific activity (µg tyrosine released /mg protein/min) of proteases of acetone (1) and ethanol (2) treated layer chicken intestine powders (n=3). Intestine homogenate to solvent ratio = 1:1 (a), 1:1.5 (b) and 1:2 (c)

and the activity of proteases. Treatment with acetone at the ratio of 1:1.5 (intestine:acetone) resulted in higher protease activity.

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