Characterization of Two Proteases from *Enterobacter* and *E. coli* Isolated from Processed Foods in the West African Sub-Region

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Abstract: Continuous study of the diversity as well as the dynamics of microbial communities in processed foods are being undertaken worldwide to check the prevalence and spread of gastrointestinal tract-related illnesses. Indigenously processed food samples of different types were collected from two Francophone countries – Togo and Benin and two Anglophone countries – Ghana and Nigeria in the West African sub- region during the wet and dry seasons of a sampling period of two years. Enterobacteria were isolated from each of the samples using standard techniques. The isolates were subjected to enzymatic screening for protease production. These enzymes were assayed and characterized. Protease activity observed demonstrates the enzymes are metallo-proteases and not serine-proteases. Acid-stable proteases (pH 5.4) were detected in the isolates. Protease activity was lowest at 20^{o}C and highest at 50^{o}C for both organisms. The presence of enteric bacteria in the foods analyzed provide undeniable evidence of the poor microbiological quality of indigenously processed foods could form the basis of a useful databank in formulation of food-borne disease control and prevention strategies in the sub-region.
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1.0. INTRODUCTION

Different enzymes involved in food degradation in the gut have been used in characterisation of the enterobacteriaceae. Enzymes such as amylases, cellulases, chitinases, proteases and DNA polymerases from extremophiles have proven to be useful for industrial processes and biotechnology procedures (Deutch, 2002). Enzymes found in the intestinal lumen could potentially have come from either the pancreas or the secretory cells in the gut wall. In addition, enzymes from the intestinal microflora potentially could have a significant role in digestion especially for substrates such as cellulose which few animals can digest and for other substrates as well (Smith, 1989).

Proteases (or subtilisins) catalyse the hydrolysis of proteins to peptides and amino acids. These enzymes are important from an industrial perspective and cater to the requirement of nearly 60% of the world enzyme market (Kalisz, 1988). Global requirements of thermostable biocatalysts are shown to be far greater than those of the mesophiles, of which proteases contribute two thirds (Ng and Kenealy, 1986). Alkaline proteases produced by alkalophiles are of interest from a biotechnological perspective and are investigated not only in scientific areas like protein chemistry and protein engineering, but also find application in detergents, food, pharmaceutical and tannery industries (Kumar and Takagi, 1999; Kumar, 2002). Most of the proteases from microorganisms are either thermostable and alkali labile or thermolabile and active at high pH values (Tsuchiya *et al.,* 1992).

Proteases are mainly derived from mesophilic sources that work in narrow ranges of pH, moderate temperatures and narrow limits of tolerance to detergents. However, alkaline proteases exhibit optimum activity and stability at high pH(s) and temperatures, surfactants and chaotrophic agents, which make them suitable candidates for industrial applications (Kumar and Takagi, 1999; Kumar, 2002).

This work is to characterise and find out the stability of proteases derived from enterobacteria isolated from ready-to-eat convenience foods.

2.0. MATERIALS AND METHODS

2.1. Sampling and description of sampling sites

The convenience food samples collected included puff-puff, egg rolls, buns, fried chicken, fish pie etc. The convenience food samples were collected from two Anglophone and two Francophone countries in the West African sub-region. These are Accra (Long. 0^{o} 09'W, Lat. 6^{o} 00'N) the capital of Ghana and Aflao (Long. 0^{0} 46'E, Lat. 6^{0} 11'N) a town on the eastern border of Ghana; Lome the Togolese capital (Long. 1^{o} 13'E, Lat. 6^{o} 07'N) and La Kondji (Long 1^{0} 40'E, Lat. 6^{0} 18'N) which is a border town east of Lome in Togo. This was followed by the capital of the republic of Benin,

Cotonou (Long. 2^o 26'E, Lat. 6^o 21'N) and Seme (Long. 2^0 37'E, Lat. 6^0 22'N) an eastern border town between the Republic of Benin and Nigeria (Figure 3.1). The fourth country was Nigeria in which sampling was carried out in some towns. The towns from which sampling was carried out in Nigeria were Port-Harcourt, Yenagoa, Abakaliki, Enugu, Ibadan, Ado-Ekiti, Ikeja, Yola, Gombe, Abuja, Makurdi, Jos, Sokoto and Birnin-Kebbi (Figure 1).

Fig. 1. Map of West Africa showing the four countries where sampling was carried out.
• Dotted spot represent sampling site.

2.2. Sampling procedures

The food samples were collected during the rainy (wet) season i.e. between April and October and harmattan (dry) season i.e. between November and March for two years 2005 and 2006. Convenience food samples were collected in clean polyethylene bags and labelled accordingly. The polyethylene bags with the samples were transported aseptically in insulated rectangular, plastic food flasks (Kenchuang Co., Xhenkong, China (30 cm x 17.5 cm x 30 cm)) filled with ice chips to the Department of Botany and Microbiology laboratory, University of Ibadan, Ibadan, Oyo State, where they were unloaded and refrigerated immediately. Whenever possible, 100g was obtained for each sample unit. The average time of travel from sampling sites outside Nigeria (Ghana through Lome and Cotonou to Ibadan) was thirteen and a half (13.5) hours. Average travel time within Nigeria was eight and a half hours

2.3. Isolation and culture methods

2.3.1. Sample treatment to obtain isolates: Twenty- five grams of each food sample was homogenised with nine times that weight or volume of buffered peptone water (Andrews and Hammack, 2002). The different food samples were treated separately. The egg roll was treated by isolating from the flour covering; the boiled egg and a combination of both. The chicken was treated by sampling from the skin and the flesh separately. The pies- meat and fish were treated in the same manner. Each sample was mixed to ensure homogeneity. A portion (1g) of the resulting homogenate was then transferred into sterile test tubes and serially-diluted ten-fold according to the methods described by Fawole and Oso (1985) and Pollack *et al.* (2002). The diluents were then plated out for incubation to get pure cultures and further work was carried out on the isolates (American Public Health Association 1992; AOAC, 1995; Baudart *et al.,* 2000; Andrews and Jacobson, 2003). The media for isolation were Nutrient Agar and Broth (Lab M), MacConkey Agar and Broth (Fluka); Salmonella-Shigella Agar (Lab M); Bismuth Sulphite Agar (Fluka); EMB Agar (Fluka). All were prepared according to the manufacturers instructions. Maintenance of Pure Cultures: Pure cultures of isolates were stored on Nutrient agar slants at 4^oC. The organisms were sub-cultured onto fresh slants every three months (90 days). The bacterial colonies were differentiated first on the basis of colonial morphology followed by microscopic examination after Gram staining. Gram staining and biochemical tests were carried out to characterise the isolates as described by Pollack *et al.,*(2002).

2.3.2. Test for Milk Digestion (Protease Production): Each test bacterium was inoculated by streaking onto solid skimmed milk agar medium (Skimmed milk powder 50g/L; Nutrient agar 28g/L). Incubation was done at 22^oC for 3 days. Proteolytic activity was noted as a zone of clearing around the stab (Rajmohan *et al.,* 2002) or after flooding the plates with Mercuric chloride solution (mercuric chloride, 15g; Conc. HCl, 20mL; distilled water, 100 mL). Unhydrolysed casein form white opaque precipitate, clear zones indicate casein hydrolysis (Olutiola *et al.,* 2000).

2.4. Protease enzyme preparation

2.4.1. Production of protease: Each isolate was grown in semi synthetic medium containing $10gL^{-1}$ casein –peptone, $5gL^{-1}$ NaCl, $3.15gL^{-1}$ Na_2HPO_4, and $1.5gL^{-1}$ NaH_2PO_4 for 24h at 37^oC (Tondo *et al.*, 2004). The culture supernatant obtained by centrifugation at 10 000g for 5min, were used as crude enzyme preparation (Tondo *et al.*, 2004). Protease activity was measured by the modified azocasein assay of Sacherer *et al*., (1994).

2.4.2. Protease Assay : Reaction mixture contained 0.5 mL azocasein (0.5% w/v) dissolved in 0.1molL^{-1} citrate buffer, pH 6, by boiling for 15min in a water bath and then filtered, 0.5 mL 0.1 molL^{-1} citrate buffer, pH 6 and 0.1 mL culture supernatant fluid (Rajmohan *et al.,*2002). The reaction mixture was incubated at 37℃ for 30min, then stopped with 1.1 mL 10% (w/v) trichloroacetic acid (TCA) and left on ice for 15min. The samples were clarified by centrifuging at 5000g for 10min and optical density readings of the supernatant fluid measured at 600nM in a Colorimeter (Jensway, Ersex, UK). One unit of enzyme activity is defined as the amount of protease required to produce an absorbance increase of 0.01 under the described assay conditions. The blank contained azocasein with citrate buffer (De Azeredo *et al.,* 2006).

2.5. Characterization of enzyme

2.5.1 Effect of pH on protease activity: this was determined with 0.1M Citrate phosphate buffer at varying pH values ranging from 5.0 -7.8. Zero point five (0.5) mL casein mixture was boiled for 15min and then filtered. Zero point five (0.5) mL of 0.1M citrate phosphate buffer (at the different test pH values) was added to 0.1 mL culture supernatant fluid and the mixture incubated at 37^{o}C for 30 min. The assay proceeded as described above.

2.5.2. Effect of inhibitors: an overnight culture was grown and shaken at 180 rev/ min and centrifuged at 5000g for 20 min. The supernatant fluid was pre-incubated at 27℃ for 1h at pH 7 with each salt EDTA and $CaCl_2$ dissolved at different concentrations (0, 5, 10, 15, 20 and 25 10 mmolL^{-1}). A protease assay was performed as described above with a control containing citrate buffer without inhibitor.

2.5.3. Effect of temperature: the effect of temperature on the activity of the enzyme was determined. The reaction mixtures (0.5 mL casein, 0.5 mL 0.1M citrate phosphate buffer and 0.1 mL culture supernatant fluid) were incubated for 30mins at 20^{o}C, 30^{o}C, 40^{o}C – 70^{o}C respectively. Protease activity was determined as described above (Patel *et al*., 1986; Lee *et al*., 1997).

3.0. RESULTS

3.1. Number, percentage occurrence and mean Enterobacterial counts.

Forty three organisms in all were isolated. Generically the organisms were *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Escherichia coli*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia* and *Shigella*. The number and frequency of occurrence are shown in Table 1, with *Enterobacter* recording the highest percentage of 27.92% while *Edwardsiella*, *Salmonella* and *Serratia* had the minimum occurrence of 4.65% each.

The mean values for isolated organisms from the four countries are shown in Figure 2. The mean count was higher in year 2005 and reduced in year 2006 for Ghana, Benin and Nigeria (Fig 2). In Togo, the mean count increased in the year 2006 being lower in the year 2005 (Fig 2).

3.2. Enzyme production

Two of the isolates produced protease enzymes, one *E. coli* and one *Enterobacter* sp. Proteolytic activity was observed by clear zones directly or after flooding the plates with mercuric chloride solution around streaks of organisms on nutrient agar plates containing skimmed milk (5%).

Table 1: Number and Percentage Occurrence of Isolates in the Different Convenience Food Samples.

ISOLATE	NUMBER	PERCENTAGE
Citrobacter sp	6	13.95
Enterobacter sp	12	27.92
Edwardsiella sp	2	4.65
Escherichia coli	4	9.30
Klebsiella sp	4	9.30
Proteus sp	4	9.30
Salmonella sp	2	4.65
Serratia sp	2	4.65
Shigella sp	7	16.28
Total	43	100

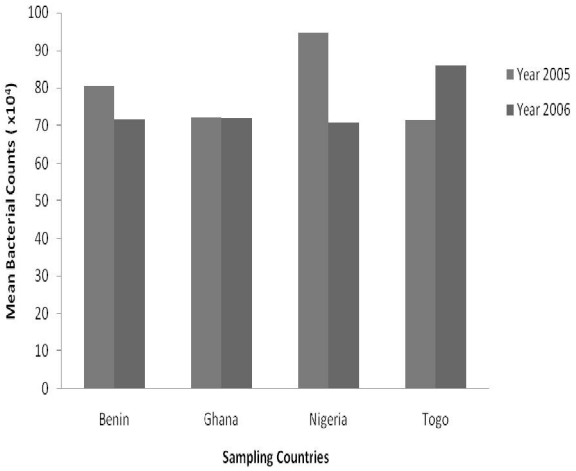

Fig.2: Mean values of Counts of Enterobacteria isolates from Samples Collected in the Four Countries within Year 2005 and 2006.

3.3. Effects of Inhibitors, pH and Incubation temperature on protease activity.

Effects of two inhibitors EDTA and $CaCl_2$ were studied on the protease extracted from *E.coli* in Figure 3. *E. coli* protease activity was 79.5units/mL at 20mM of $CaCl_2$ but the activity fell drastically to 36.5units/mL at 25mM. Protease activity was on the average at 5mM of EDTA. The activity fell from 52units/mL at 10mM to 43units/mL at a concentration of 15mM of EDTA. The activity was highest (61units/mL) at 25mM EDTA (Figure 3). *Enterobacter* protease activity was highest at the inhibitory concentration of 20mM for both EDTA and $CaCl_2$. Protease activity fell to 67units/mL for EDTA and 37units/mL for $CaCl_2$ at 20mM concentration of both inhibitors. The inhibitory effect of $CaCl_2$ was higher than that of EDTA (Figure 4).

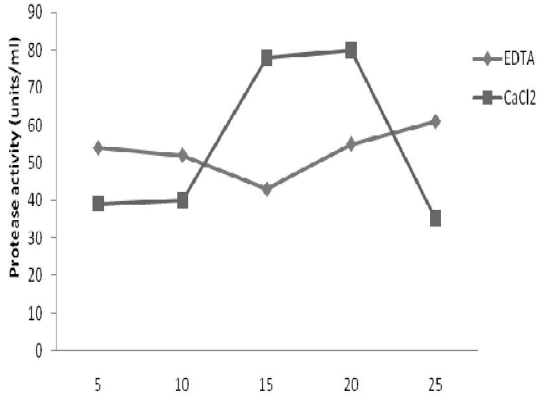

Figure 3: Effects of some inhibitors on the protease activity of *Escherichia coli* isolated from processed foods.

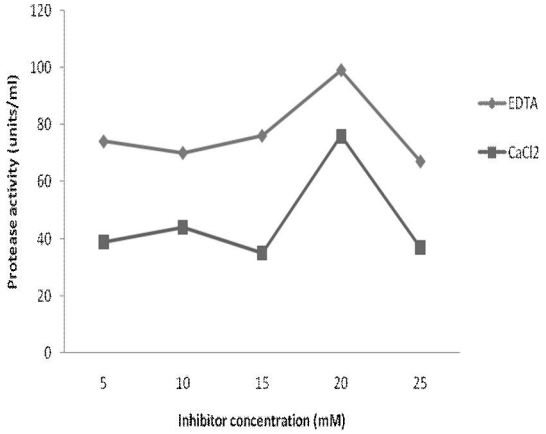

Figure 4: Effects of some inhibitors on the protease activity of *Enterobacter* isolated from processed foods.

Optimum pH for protease activity for both *E. coli* and *Enterobacter* was 5.4. Activity was lowest at pH 7.8 for both bacteria. *Enterobacter* protease activity (96units/mL) was higher than *E. coli* protease activity (89units/mL) at the optimum pH 5.8 (Figure 5).

Figure 6 shows the effect of incubation temperature on protease activity of *E. coli* and *Enterobacter*. Protease activity was lowest at 20°C for both organisms. *E.coli* activity was 31units/mL while *Enterobacter* activity was 30units/mL. Highest activity for *Enterobacter* (84 units/mL) was recorded at 50°C while the highest activity was 81 units/mL for *E.coli*. Protease activity fell to less than 60% at 70°C with *Enterobacter* protease being more heat labile (52 units/mL) than the protease from *E. coli* (55 units/mL).

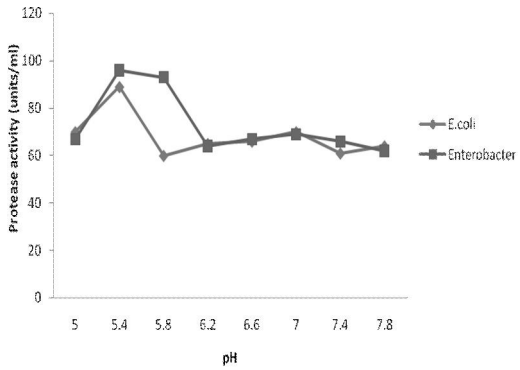

Figure 5: Effects of pH on the protease activity of *E. coli* and *Enterobacter* isolates from processed foods

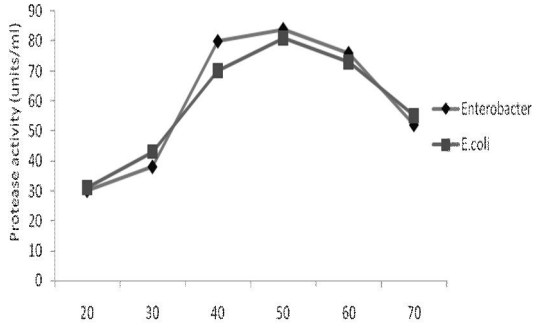

Figure 6: Effects of temperature on the protease activity of *E. coli* and *Enterobacter* isolates from processed foods

4.0. DISCUSSION

In this study, protease was secreted by *E. coli* and *Enterobacter* species. A psychrotrophic proteolytic strain of *Klebsiella oxytoca* was isolated from raw milk and characterised (Tondo *et al.* 2004). *K. oxytoca* as well as *E.coli* and *Enterobacter* are coliforms. They can be isolated from animal faeces, vegetables and pathological processes, and from aquatic environments (Orskov, 1984). They have also been isolated from food samples (Lawal, 2008; Owoseni and Onilude, 2011), including milk (Singh *et al.* 1996). Proteases secreted by strains of *Pseudomonas fluorescens* isolated from cow's milk have been extensively studied as a key factor in causing spoilage of both pasteurized and ultra heat treated (UHT) milk (Rajmohan *et al.,* 2002).

The protease activity observed in the current study is not typical of previously identified enzyme of *P. fluorescens* with inhibition of activity by EDTA thus demonstrating that the enzyme is a metallo-protease but not a serine-protease (Rajmohan *et al.,* 2002). The *E. coli* protease activity increased with higher concentration of EDTA in this study while that of *Enterobacter* was inhibited by increasing concentration of EDTA, this suggests a metallo-protease. The protease produced by *Microbacterium* sp. was inhibited by EDTA at a concentration of 5mM (Thys *et al.,* 2004). Thys *et al.* (2004) reported that the protease of *Microbacterium* was not inhibited by $CaCl_2$ at a concentration of 5mM. The proteases studied in this work were inhibited by $CaCl_2$ at a concentration of 20mM.

Although *Pseudomonas* spp are the most frequent Gram-negative psychrotrophic micro-organisms found in raw and pasteurized milk (Cousin, 1982), psychrotrophic Enterobacteriaceae are also commonly isolated (Muir, 1996). The optimal pH for the enzymes studied here was 5.4 and the optimal temperature was 50^{o}C. Many reports explained the problems caused by thermal-resistant proteases and lipases in milk processing (Craven and Macauley 1993; Boor *et al*., 1998), but only few characterized specific microorganisms and their respective enzymes. Fernandez *et al*. (1999) and Koka and Weimer (2000) characterised proteases produced by strains of *P. fluorescens* that hydrolysed both alpha ($\alpha s_{1)}$, Beta (β) and Kappa (κ) caseins (Tondo *et al*., 2004). The multiple pH optima in the *Enterobacter* protease isolated in this work probably indicates the presence of more than one protease (Loci, 1989). The production of multiple proteases is a common feature for several streptomycetes (James *et al.,* 1991).

The presence of enteric bacteria in the foods analyzed provide undeniable evidence of the poor microbiological quality of indigenously processed foods could form the basis of a useful databank in formulation of food-borne disease control and prevention strategies in the sub-region.

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