Research Notes

Chicken Intestinal Aminopeptidase: Partial Sequence of the Gene, Expression and Activity[1]

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ABSTRACT Aminopeptidases are members of a membrane-bound metallopeptidase family that are expressed at a high level on the brush-border membrane of enterocytes. Because the rapid growth of meat-type chickens depends on the dietary supply of amino acids, a study of intestinal aminopeptidases, which play a central role in protein digestion, is important. This study is the first reported isolation of the partial cDNA of chicken intestinal aminopeptidase and sequencing of a 1.7-kb cDNA fragment. The gene was isolated by reverse transcriptase polymerase chain reaction using six primers chosen from conserved regions of the aminopeptidase genes. Amplified fragments were extracted from the gel, purified, and sequenced. By using this chicken cDNA as a probe, northern blot analysis revealed a transcript of approximately 3.5 kb in the chicken duodenum, jejunum, and ileum tissues. Higher RNA expression and activity of aminopeptidase were found in the ileum tissue compared with the duodenum and jejunum segments.

(*Key words*: chicken, aminopeptidase, small intestine, nucleotide sequence, polymerase chain reaction)

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INTRODUCTION

Aminopeptidases (APN) are enzymes that belong to a group of exopeptidases and include 1) APN enzymes, such as EC 3.4.11.1, EC 3.4.11.2, and EC 3.4.11.3, which cleave peptides preferentially after the N-terminus of neutral amino acids; 2) enzymes such as EC 3.4.13.8, which are specific for dipeptide substrates; and 3) enzymes such as EC 3.4.15.1, which split off dipeptide units from the C-terminus (Maroux *et al.*, 1973; Feracci and Maroux, 1980).

The APN enzymes are anchored to the membrane *via* an N-terminal hydrophobic sequence of 20 amino acids that span the membrane only once (Feracci and Maroux, 1980; Feracci *et al.*, 1982; Maroux and Feracci, 1983). The cytoplasmic domain is very short, and nearly the entire molecule is located on the cell surface. Aminopeptidase has been purified from rat skin (Jarvinen and Hopsu-Hava, 1975), rat liver and kidney (Kirshchke *et al.*, 1976; Watt and Cecil, 1989), rabbit lung (Singh and Kalnitsky, 1980), and chicken skeletal muscle (Rhyu *et al.*, 1992). Intestinal APN cDNA has been cloned and sequenced from nematodes (Smith *et al.*, 1997), rabbits (Noren *et al.*, 1989), and humans (Olsen *et al.*, 1988). The molecular characterization of APN from chicken egg yolk and liver has been recently published (Midorikawa *et al.*, 1998; Adachi *et al.*, 1997). However, in chickens, intestinal APN activity, expression, and cDNA sequence have not been reported.

Because the rapid growth of meat-type chickens partially depends on the dietary supply of amino acids, a study of the intestinal APN that play a central role in protein digestion is important. The isolated cDNA fragment covers almost 60% of the gene, including the Zn-binding region. The primary structure of the protein was deduced from the cDNA sequence. The fragment was used as a probe for measuring RNA expression in the duodenum, jejunum, and ileum of chicken. In addition, enzyme activity was examined in these three regions of the small intestine.

MATERIALS AND METHODS

Intestine tissues were taken from five Arbor Acres chickens that were killed at 28 d of age with an intracardiac overdose of sodium pentobarbital (0.2 g/kg). The small intestine was removed and ~2.5-cm segments were dissected from duodenum, jejunum, and ileum. Initial preparation involved flushing all tissue samples with 0.9% NaCl to remove the intestinal contents. The pieces

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Abbreviation Key: RT-PCR = reverse transcriptase polymerase chain reaction; APN = aminopeptidase.

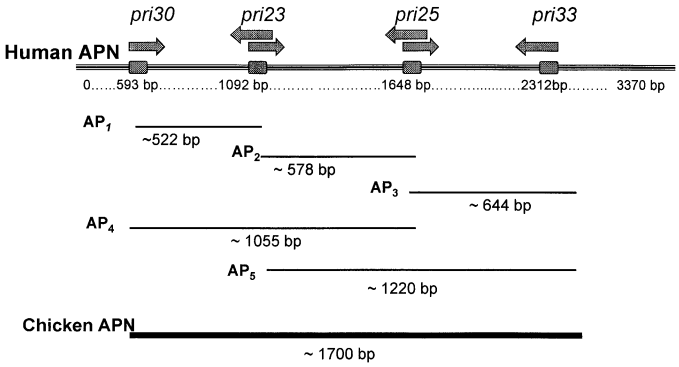

FIGURE 1. Polymerase chain reaction (PCR) cloning strategy. The primers and location are shown with arrows. The six PCR products of chicken aminopeptidase cDNA that were isolated and sequenced are indicated. APN = Aminopeptidase.

of tissue were then routinely frozen in liquid nitrogen and stored at –80 C until further analysis.

Total RNA was isolated from the tissues using TRI REAGENT[3] (1 mL/100 mg tissue) according to the manufacturer's protocol.[3] The RNA from the five chickens served as the source for the reverse transcriptase-polymerase chain reaction (RT-PCR). A comparison of five different published sequences of the APN gene from different sources—rat kidney (GenBank/EMBL M25073), mouse mast cell (GenBank/EMBL U77083), rabbit kidney cortex (GenBank/EMBL S68687), rabbit intestine (GenBank/EMBL X51508), and human intestine (GenBank/EMBL X13276)—enabled us to identify common regions. The six primers, chosen from four conserved regions were pri30 (forward) 5′-TGGC GGGCTTCTACCGCAGCGAG-3′; pri23 (forward) 5′-CTGGGGACTGGTGACCTACCGGG-3′; pri23 (reverse) 5′-CCCGGTAGGTCACCAGTCCCCAG-3′; pri25 (forward) 5′-CGCTGGACCCTCGAGATGGGCTT-3′; pri25 (reverse) 5′-AAGCCCATCTCGAGGGTCCAGCG-3′; pri33 (reverse) 5′-TTGGAGCAGGCGGTGCTGATGGC-3′. Primer orientation and location in the human AP cDNA sequence (GenBank/EMBL X13276) are shown in Figure 1.

The cDNA was amplified using the Promega Access RT-PCR System[4] according to their technical bulletin #TB220: 2 min at 94 C, 30 s at 60 C, and 2 min at 68 C for 36 cycles, followed by 7 min at 68 C.

The RT-PCR products were run on a 1.5% agarose gel, visualized by staining with ethidium bromide, excised from the gel, and purified with a gel extraction column (Wizard™ PCR Preps[4]). The chicken APN cDNA fragment and the five independently isolated overlapping cDNA fragments—AP_1, AP_2, AP_3, AP_4, and AP_5—were subjected to automated sequencing using an Applied Biosystem 373A DNA sequencer.[5] Nucleic acid sequences were analyzed using the GCG suite of programs (Devereux *et al.*, 1984) on a VAX 4000-300 computer. The homology between chicken and other APN sequences was calculated using DNAMAN version 4, Lynnon Biosoft© 1994 to 1997.

For northern blot analysis, 30 μg of total RNA was denatured and separated by electrophoresis through a 1.5% agarose/1.1 *M* formaldehyde gel. After electrophoresis, RNA was capillary transferred overnight to a nylon filter[6] and then fixed on the filter by baking. Prehybridization (42 C), hybridization (42 C), and washing (57 C) were conducted according to the procedures recommended by Amersham[6] for Hybond N+ membranes. The 522-bp cDNA fragment (AP_1) was labeled with ^{32}P-dCTP by the random prime labeling method (Promega[4]) and was used as a probe. After a high-stringency wash (0.1× saline sodium citrate/0.1% SDS at 57 C) blots were exposed for 24 h at –70 C to Kodak XAR 5 film[7] in the presence of an intensifying screen.

Aminopeptidase activity in the small intestine was assayed in homogenized duodenal, jejunal, and ileal tissues (250 mg tissue/5 mL of 50 mM sodium phosphate buffer, pH 7.2) from five 28-d-old chickens. Determination of the APN (EC 3.4.11.2) activity is based on the hydrolysis of the substrate L-leucine-*p*-nitroanelide (Sigma[8] L-9125) to *p*-nitroanelide and L-leucine by the APN in the homogenate. Reaction time was 15 min at 37 C. The *p*-nitroaniline is determined by staining and measuring spectrophotometrically the intensity of the color at 405 nm. Aminopeptidase activity is expressed in units per gram of intestinal tissue protein. One unit of APN activity is equal to the production of 1 μmol of *p*-nitroanilide/min from the L-leucine-*p*-nitroanilide substrate. This method for determination of APN activity has been described previously (Maroux *et al.*, 1973). Total protein was determined using Bio-Rad protein assay[9], a colorimetric assay for protein concentration following detergent solubilization.

RESULTS AND DISCUSSION

The 1680-bp cDNA fragment of chicken intestinal APN showed 72% identity to the human and rabbit intestinal APN sequences and only 46% identity to the nematode intestinal sequence. The sequence was 99% identical to that of chicken egg yolk APN (Midorikawa *et al.*, 1998) and contained an open reading frame yielding a predicted translation product of 560 amino acids. The deduced chicken intestinal APN includes nine possible Asn-X-Ser/Thr N-glycosylation sites and the functional conserved sequence, the HEXXH domain, which is rich in potential Zn^{2+} ligands. The predicted amino acid sequence (Figure 2) was 73.4% identical over the 560 amino acids to human intestinal APN, 73.1% identi-

[3]TRI-REAGENT Molecular Research Center, Inc., Cincinnati, OH 45212.
[4]Promega Corp., Madison, WI 53711-53990.
[5]Weizman Institute, Rehovot, Israel 76100.
[6]Amersham, Arlington Heights, IL 60005.
[7]Eastman Kodak Company, Rochester, NY 14650.
[8]Sigma, Rabin Park, Rehovot, Israel 76100.
[9]Bio Rad, Hercules, CA 94547.

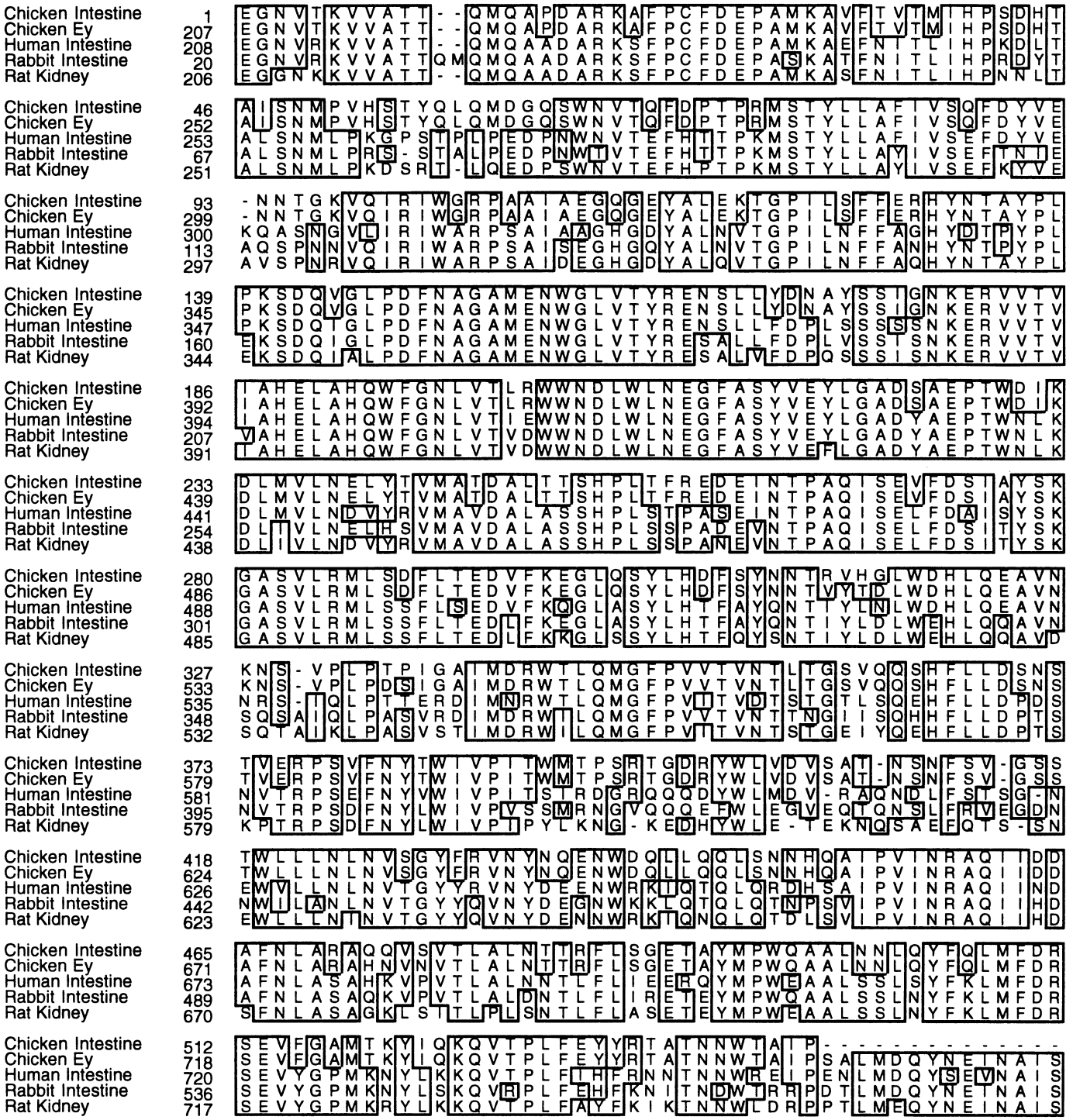

FIGURE 2. Alignment of predicted amino acid sequence of the intestinal aminopeptidase chicken gene and other aminopeptidase amino acid sequences. Conserved sequences in at least four sequences are shaded. Chicken Ey = chicken egg yolk (GenBank/EMBL 87992.gb_ov). The sequence data reported in this paper have been submitted to the GenBank Data Library under the accession number Y17105.

cal to rabbit intestinal aminopeptidase, and 68% identical to rat kidney APN.

By using the AP_1 cDNA fragment as a probe, northern blot analysis revealed a transcript of approximately 3.5 kb in the jejunal, ileal and duodenal tissues (Figure 3). Other cDNA fragments (AP_2 and AP_3) used as probes detected the same size transcript (data not shown). Higher expression (Figure 3) and activity (Figure 4) of APN was demonstrated in the ileum. Similar levels of activity (80 to 150 U/g protein) were found in the pig and rat small intestine (Van Leeuwen *et al.*, 1995; Kaur *et al.*, 1996). This level differs from the expression and activity of the sucrase-isomaltase brush-border enzyme, which was lowest in the duodenum, highest in the jejunum, and intermediate in the ileum (Uni, 1998; Uni *et al.*, 1998). The methodology of isolating a specific

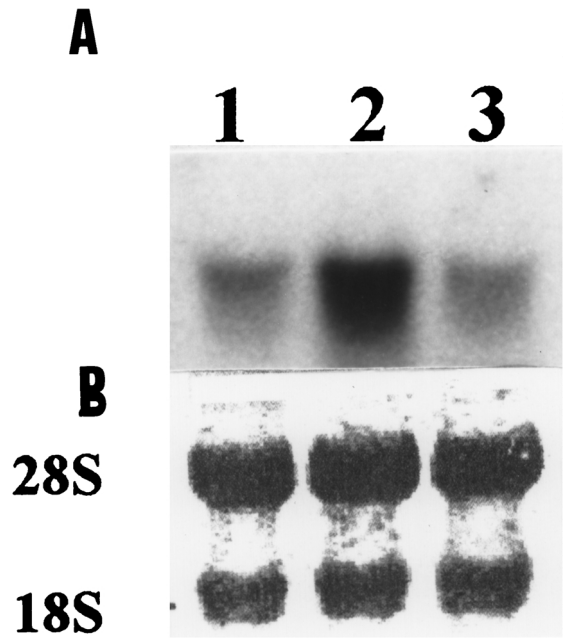

FIGURE 3. Northern blot analysis of aminopeptidase expression in three segments of the chicken small intestine. Panel A shows the autoradiography of the northern blot analysis of 30 μg of total RNA from chicken intestinal tissues hybridized to the chicken AP_1 cDNA probe. 1 = jejunum; 2 = ileum; 3 = duodenum. Panel B shows ethidium bromide-stained gels of RNA samples as a control for RNA integrity.

cDNA fragment using RT-PCR and primers from conserved regions in a gene has been used in our laboratory previously, enabling us to partially isolate the sucrase-isomaltase gene from chicken enterocyte cells (Uni, 1998). In this present work, we did not use primers from the APN cDNA sequence isolated from chicken liver (Adachi *et al.*, 1997) because very low homology (35%) was found between it and other published APN sequences (rat kidney, mouse mast cell, rabbit kidney cortex, rabbit intestine, and human intestine). Moreover, we were not able to use the sequence information published by Midorikawa *et al.* (1998) because we performed our study prior to its publication.

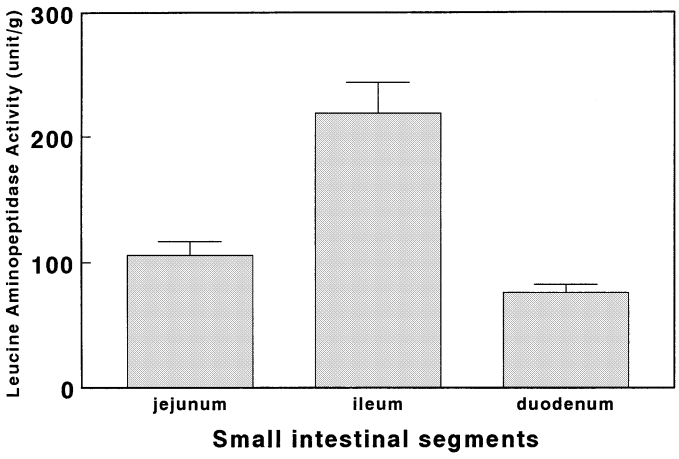

FIGURE 4. Activity of aminopeptidase per 1 g of intestinal protein in the jejunum, ileum, and duodenum of 28-d-old broiler chickens. Results are means of five chickens and bars are SD.

The current isolation of a 1680-bp chicken intestinal APN cDNA will provide a new tool for future studies on the expression of the APN enzyme. Using this intestinal chicken cDNA fragment as a probe will enable us to map the APN gene to a chromosome as well as to investigate polymorphisms between chicken strains. In addition, it will be possible to examine the expression and activity of intestinal APN during different phases of growth and under different dietary protein levels and to study the correlation between protein digestibility and APN activity.

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