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Chicken intestine: A source of aminopeptidases

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ABSTRACT: The suitability of chicken intestine, a poultry processing by-product, as a source of aminopeptidases has been evaluated. To investigate the heterogeneity of the aminopeptidases in the tissue, tissue fractions separated by differential centrifugation and ion exchange chromatography were screened for aminopeptidase activity using twelve different amino acid naphthylamide substrates. The sedimentable fractions obtained by differential centrifugation, although differing in substrate profile, were largely enriched (72–90%) in aminopeptidase activities. However, the enzymes hydrolysing pro-β-naphthylamide largely belonged to the soluble fraction ($>$ 40%). On the basis of their interaction with the ion exchange resins, DEAE and CM Sepharose, six regions of aminopeptidase activity differing in their elution profiles and substrate specificities were identified. Data reveal the presence of a number of aminopeptidases with multiple specificities in chicken intestine which could be used for industrial applications.

KEYWORDS: poultry viscera, proteases, by-products

INTRODUCTION

Aminopeptidases are exopeptidases that catalyse the hydrolysis of peptide bonds from the N-terminus of proteins and peptides[1]. They have a diverse distribution among prokaryotes and eukaryotes[2] with broad and overlapping substrate specificities. Aminopeptidases are classified in terms of their affinity to N-terminal amino acids, response to inhibitors, and localization in subcellular compartments[3]. Apart from their role in key physiopathological reactions in the cell[4], aminopeptidases have many important commercial applications. The food industry uses them for debittering and improving the functional properties of protein products[5] and for flavour development in cheese[6]. In the pharmaceutical industry, inhibitors developed against these enzymes find application as drugs[7]. Aminopeptidases are also used for peptide sequencing[8] and in processing of recombinant proteins[9]. Today the industrial demand for aminopeptidases far exceeds their availability and the need to identify new sources is ever increasing[10,11]. Aminopeptidases have been isolated and characterized from various tissues. However, the content of individual aminopeptidase in these sources is not very high[12] and so it is difficult to use these materials as a source of these enzymes. However, certain animal tissue organs such as the pancreas and intestine are rich source of exopeptidases exhibiting activity high enough for industrial applications[12]. We have shown that chicken intestine, an underutilized by-product of poultry industry is a rich source of endo- and exopeptidases[13,14]. We have developed a process for the debittering of commercial protein hydrolysates using chicken intestinal aminopeptidases[15]. Although Gal-Garber et al[16] have reported the activity of Leu-aminopeptidase in the chicken intestinal segments (ileum, jejunum, and duodenum), a comprehensive study with respect to different types of aminopeptidases, their substrate specificity, and cellular location has not been reported. Here we attempt to evaluate the total profile of aminopeptidases in this tissue with a view to using it as a commercial source.

MATERIALS AND METHODS

Materials

p-chloromercuribenzoate (PCMB), Brij-35, Fast Garnet GBC, cysteine and the β-naphthylamide derivatives of L-Arg, L-Ala, L-Leu, L-Tyr, L-Phe, L-Trp, L-Val, L-Gly, L-His, L-Ser, L-Asp, and L-Pro were obtained from Sigma (St. Louis, MO). The ion exchange resins DEAE sepharose and CM sepharose were products of GE Healthcare. All other reagents were of analytical grade.

Procuring and pre-processing of chicken intestinal tissue

Chicken intestine was brought in ice from a local abattoir soon after slaughter and freed of other organs such as spleen and pancreas along with the overlying layer of fat and connective tissue. The undigested food

and faecal matter were flushed out with tap water and the tissue was held at 0–4 °C until further processing.

Peptidase assay

The assay of aminopeptidase activity was carried out as reported previously[14]. A 1 ml reaction mix consisting of the enzyme, 0.1 M sodium phosphate buffer (pH 6.8), 1 mM cysteine, and 1 mM amino acid β-naphthylamide was incubated at 50 °C. The reaction was terminated by the addition of 1 ml Fast Garnet GBC-PCMB reagent which consisted of 0.2 mg/ml Fast Garnet GBC (in 4% Brij 35) and 10 mM PCMB reagent mixed equally just before terminating the assay. The pink coloured β naphthylamine-GBC complex was measured at 520 nm.

Preparation of tissue homogenates

Homogenates were prepared from intestine, mucosa, and wall. Mucosa was separated by scraping the longitudinally opened sections of the intestine with a spatula. The walls were thoroughly washed with tap water to remove any remaining part of the mucosa. 40% (w/v) homogenates were separately prepared from the wall, mucosa, and intestines in 10 mM sodium phosphate buffer (pH 6.8) containing 0.1% Triton X 100 by grinding the tissue in a domestic grinder (30 s × 3) followed by three bursts of 30 s each in a Polytron homogenizer (Kinemaica) at a setting of 4.

Sub-cellular fractionation

A 20% homogenate of chicken intestine in 0.25 M sucrose (adjusted to pH 7.0 with 0.1 M Tris) was prepared as above. The homogenate was filtered through a double layer of muslin cloth and was centrifuged at 750g for 10 min to obtain the nuclear fraction. The supernatant was further centrifuged at 3000g for 10 min to obtain the pellet of mitochondrial fraction and the lysosomes and microsomes suspended in the cytosol. The lysosomes were separated from this mixture by centrifuging at 17 300g for 15 min. The cytosolic and microsomal fractions were obtained by ultra centrifugation (Beckman Instruments) at 100 000g for 1 h. The identity of the sub-cellular fractions was ascertained by the assay of marker enzymes[17]. Aliquots of the individual fractions were diluted 1:10 with distilled water containing 0.1% Triton-X-100. These aliquots were used for the assay of aminopeptidase activity with the β-naphthylamide substrates as mentioned above. A freeze and thaw of the fractions prior to aminopeptidase assay was not performed as some of these enzymes (e.g., puromycin-sensitive aminopeptidase) lose activity on the freeze and thaw steps[14].

Ion exchange chromatography

The complete intestinal homogenate (40%) was centrifuged at 34 800g for 40 min. The supernatant was filtered through a double layer of muslin cloth. One half of this supernatant was used for DEAE Sepharose chromatography. The pH of the other half of the supernatant was adjusted to 5.5 and centrifuged at 17 300g for 15 min. This supernatant was used for CM Sepharose chromatography. The absorbance at 280 nm and the NaCl concentration of the individual fractions eluted from the columns was monitored.

For anion exchange chromatography, the intestinal supernatant, prepared as above (pH 6.8) was applied to a DEAE Sepharose column equilibrated with 5 mM sodium phosphate buffer (pH 6.8) and unbound proteins were removed by washing with two column volumes of equilibrating buffer. Bound proteins were eluted by a linear gradient from 0–0.5 M NaCl in equilibrating buffer. The aminopeptidase activity of every third fraction was assayed against all the β-naphthylamide substrates.

For cation exchange chromatography, the intestinal supernatant (pH 5.5) was applied to a CM Sepharose column equilibrated with 5 mM sodium dihydrogen phosphate (pH 5.5) and unbound proteins were removed by washing with two column volumes of equilibrating buffer. Bound proteins were eluted by a linear gradient from 0–0.5 M NaCl in equilibrating buffer. The aminopeptidase activity of every third fraction was assayed against 13 β-naphthylamide substrates.

ESTIMATION OF PROTEIN CONTENT

The protein content of the samples was estimated by the Miller's modification of Folin Lowry method[18].

Statistical analysis

Two complete intestines were used for the tissue separation, subcellular fractionation, and for ion exchange separation. Each assay was performed in duplicate. The values reported are a mean of the values of three independent experiments. The mean and standard error (SE) were computed using ORIGIN 6.0 PRO.

RESULTS AND DISCUSSION

Observations outlined here clearly indicate that chicken intestine, especially the mucosal tissue, is a rich source of aminopeptidases which could be easily separated by aqueous extraction (Table 1). Studies

Table 1 Specific activity and yield of aminopeptidase activities in the mucosa and wall of chicken intestine.

Amino Acid NA[a]	Specific activity (μmol/mg/h)	
	Wall	Mucosa[b]
Phe	4.94 ± 0.10	15.76 ± 1.24 (87.77 ± 0.05)
Ala	5.55 ± 0.41	14.34 ± 0.07 (82.53 ± 2.77)
Leu	5.32 ± 0.40	13.50 ± 0.78 (84.96 ± 2.77)
Tyr	3.46 ± 0.33	11.80 ± 0.13 (88.47 ± 1.50)
Arg	4.17 ± 0.27	5.34 ± 0.22 (69.95 ± 2.68)
Trp	1.70 ± 0.44	3.16 ± 0.14 (77.62 ± 5.99)
Gly	0.94 ± 0.09	3.46 ± 0.10 (89.27 ± 1.33)
Val	2.12 ± 0.19	2.55 ± 0.30 (72.96 ± 1.86)
His	0.80 ± 0.07	2.34 ± 0.35 (86.31 ± 3.61)
Ser	1.06 ± 0.08	1.56 ± 0.14 (76.62 ± 4.13)
Pro	0.31 ± 0.01	0.54 ± 0.01 (79.61 ± 0.13)
Asp	0.10 ± 0.03	0.18 ± 0.05 (75.76 ± 4.40)

[a] NA = β-naphthylamine

[b] Figures in parentheses represent the yield based on total activity of mucosa + wall = 100%. All values are a mean of 3 values ± SE.

involving tissue fractionation and ion exchange chromatography point to the heterogeneous behaviour of chicken intestinal aminopeptidases.

Table 1 presents data on the relative distribution of aminopeptidases in the mucosal and the wall tissues of chicken intestine. In terms of yield, nearly 69–89% of aminopeptidases were associated with the mucosal tissue. This could be due to the presence of the brush border membranes that are rich in exopeptidases[19]. Although both mucosal and wall enzymes hydrolysed all the substrates tested, some differences in relative specificity towards individual N-terminal amino acid residues were discernible. The mucosal aminopeptidases preferred Phe, Ala, Leu, Tyr, and Arg whereas the wall enzymes were more active against Ala, Leu, Phe, and Arg β-naphthylamide. A relatively higher specificity of these enzymes towards hydrophobic amino acids also highlights their potential to be used in debittering of protein hydrolysates. In fact, taking advantage of the abundant and variety of aminopeptidase activities present in chicken intestinal mucosa (Table 1), we have already established a process for debittering of protein hydrolysates using immobilized mucosal peptidases[15].

Considerable heterogeneity was also apparent with respect to the sub-cellular localization of the enzymes (Table 2). Between 72–90% activities against Arg, Ala, Leu, Tyr, Phe, Trp, Val, Gly, His, Ser, and Asp were associated with the sedimentable fractions whereas that hydrolysing Pro (∼ 43%) was localized in the cytosolic fraction. Similar localization of aminopeptidase activities in sedimentable membrane fractions has also been reported in murine[20] and human[21] tissues. Among the membrane bound organelles, the nuclear fraction showed 15–33% activity which could be attributed to nuclear aminopeptidases[22] although some contribution by unbroken cells and brush borders[23] cannot be ruled out. The microsomal fraction possessed 16–33% activity against all the amino acid naphthylamide substrates, except Asp β-naphthylamide (∼ 10.4%). These activities could be ascribed to aminopeptidase N and aminopeptidase A, which are reported to occur in microsomes and exhibit a preference towards neutral and acidic residues[1]. The lysosomal fraction was particularly rich in Val hydrolases (30%) whereas the mitochondrial fraction exhibited 11–25% activity against all the substrates with no particular preferences. The non-sedimentable fraction (cytosol) showed enrichment in enzymes hydrolysing Arg, Ser, and Pro. The tissue fractionation results, in general, point to the particulate heterogeneity of aminopeptidases in chicken intestine which closely resembles that observed in human and rat brain[23,24], human placenta[25], and developing rat cerebellum[26].

The heterogeneity of aminopeptidases is also apparent in terms of their interaction with ion exchange resins (Figs. 1 and 2). About 70% of the aminopeptidase activity was bound to DEAE sepharose while 30% exhibited affinity for CM sepharose (data not shown). The DEAE bound aminopeptidases resolved into 2 major zones of activity (Fig. 1a,b). Zone 1 (fractions 7–14) catalysed the hydrolysis of Ala, Leu, Phe, Tyr, Gly, Trp and His. Zone 2 (fractions 19–22) exhibited preference for Ala, Leu, Trp, His, Arg, Phe, and Val. Apart from these, two minor peaks of activity (Fig. 1b) hydrolysing Ser (fraction 16) and Ser and Gly (fraction 24) were also obtained. The proline naphthylamidase showed a broader distribution encompassing fractions 12–24 (Fig. 1c). The enzyme bound to CM sepharose eluted as a single zone (Fig. 2) hydrolysing Tyr, Leu, Phe, Arg, Ala, Trp, His, Ser, Gly, and Val. The differences in the substrate specificities of the peaks and differences in elution patterns (Fig. 1) suggest the presence of multiple aminopeptidases with varying substrate specificities. Similar separation of aminopeptidases on anion exchangers has been reported from skeletal muscle of rat[27] and chicken[28]. In our laboratory, two aminopeptidases corresponding to zone 1 and zone 2 of DEAE (Fig. 1) have been purified and characterized[14,29].

To the best of our knowledge this is the first instance of screening of aminopeptidase activities in chicken intestine with respect to their distribution in tissue fractions and their interaction with ion exchange

Table 2 Percentage yields of aminopeptidases in subcellular fractions.

NA[a]	Nuclear[b]	Mitochondrial[b]	Lysosomal[b]	Microsomal[b]	Cytosolic[b]
Arg	17.8 ± 2.3	11.2 ± 0.7	17.8 ± 2.4	24.9 ± 5.3	28.4 ± 4.7
Ala	17.1 ± 3.3	14.2 ± 3.2	19.8 ± 1.8	29.3 ± 5.8	19.6 ± 1.2
Leu	22.4 ± 6.4	15.1 ± 3.4	24.3 ± 1.7	27.9 ± 5.4	10.3 ± 2.7
Tyr	22.8 ± 0.7	14.8 ± 1.3	21.2 ± 0.7	33.2 ± 1.1	8.1 ± 1.2
Phe	17.5 ± 2.7	14.0 ± 2.4	22.9 ± 0.8	32.2 ± 2.5	13.4 ± 1.9
Trp	24.1 ± 2.2	13.9 ± 1.8	20.1 ± 0.1	27.7 ± 3.2	14.3 ± 0.7
Val	27.2 ± 3.7	16.3 ± 6.6	30.4 ± 3.5	16.7 ± 3.2	9.5 ± 3.7
Gly	19.8 ± 0.8	11.9 ± 2.9	19.0 ± 1.6	31.8 ± 1.1	17.5 ± 1.1
His	32.6 ± 7.0	14.1 ± 3.4	9.5 ± 6.6	24.1 ± 1.1	19.8 ± 4.1
Ser	18.2 ± 0.3	10.3 ± 1.6	12.8 ± 2.8	27.0 ± 0.7	31.8 ± 1.6
Asp	32.5 ± 5.2	25.5 ± 1.6	18.2 ± 1.8	10.4 ± 0.2	13.4 ± 5.2
Pro	15.4 ± 4.0	11.3 ± 2.3	13.5 ± 1.3	16.2 ± 0.6	43.7 ± 5.5

[a] NA = β-naphthylamide.
[b] Values represent % yield of the fractions based on the sum of activities of nuclear, mitochondrial, lysosomal, microsomal, and cytosolic fractions = 100%. Values are a mean of three independent values ± SE.

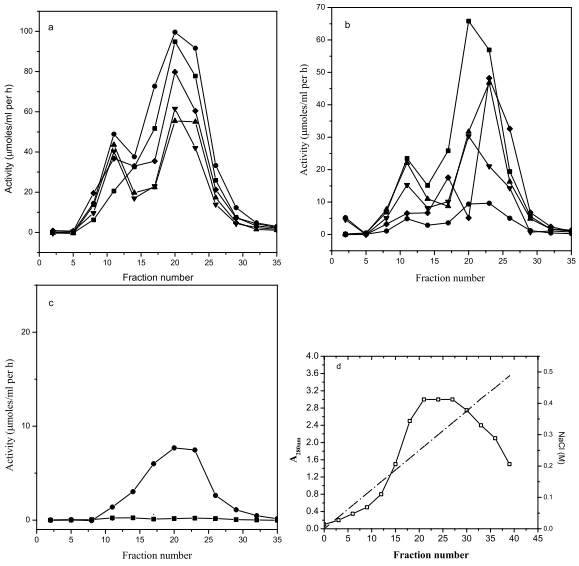

Fig. 1 Separation profile of chicken intestinal aminopeptidases on DEAE Sepharose. The separation of aminopeptidases was carried out on DEAE Sepharose column. Every third fraction was assayed for aminopeptidase activity using 13 amino acid NA substrates. (a) Arg-NA (-■-), Ala-NA (-●-), Leu-NA (-▲-), Tyr-NA (-▼-) and Phe-NA (-♦-). (b) Trp-NA (-■-), Val-NA (-●-), Gly-NA (-▲-), His-NA (-▼-) and Ser-NA (-♦-). (c) Asp-NA (-■-), Pro-NA (-●-). (d) $A_{280\,nm}$ (-□-), NaCl concentration (-··-).

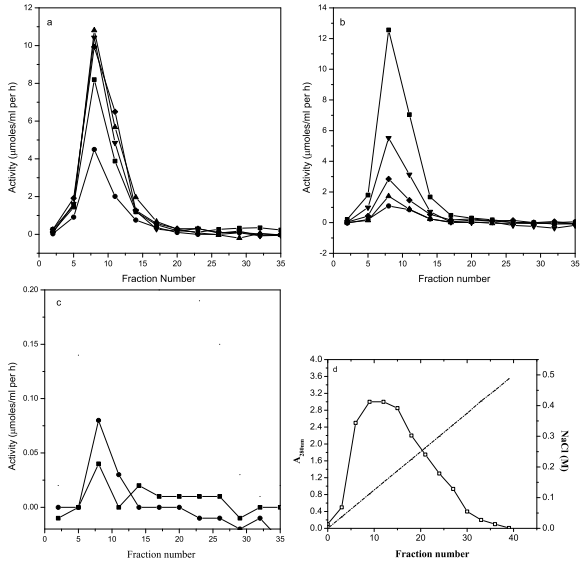

Fig. 2 Separation profile of chicken intestinal aminopeptidases on CM Sepharose. The separation of aminopeptidases was carried out on CM Sepharose column. Every third fraction was assayed for aminopeptidase activity using 13 amino acid NA substrates. (a)–(d) as in Fig. 1.

resins using an extensive array of substrates. The results outlined in this paper will be useful for preparing aminopeptidase enriched fractions or isolation of individual aminopeptidase from this tissue. Being a cheap and abundantly available by-product, chicken intestine could serve as a potential commercial source of aminopeptidases for their use in industry.

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