Purification and properties of alanine aminopeptidase from water buffalo kidney

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Abstract: Aminopeptidases participate in the development of flavour in food products. The present study aims at production of aminopeptidase(s) from the safe mammalian locally available rich sources. Three forms of alanine aminopeptidase AAP1, AAP2 and AAP3 isoenzymes were purified to homogeneity from the kidney cortex of water buffalo. The purification procedures involved anion exchange chromatography on DEAE-cellulose column and gel filtration through Sephacryl S-300 column. All of the purified isoenzymes turned out to be homogeneous as judged by native polyacrylamide gel electrophoresis. The molecular weights of the native isoenzymes AAP1, AAP2 and AAP3 were determined by gel filtration to be 120, 400 and 310 kDa. AAP1 was a homodimer of 60 kDa subunits. AAP2 was a homohexamer of 67 kDa subunits. AAP3 was a homohexamer of 53 kDa. AAP1, AAP2 and AAP3 displayed their maximum activity at pH 8, 7.8 and 7.8 and their isoelectric point (pI) values at pH 6.4, 6.2 and 6.6 respectively. The type of inhibition of AAP1 by dithiothreitol and AAP2 and AAP3 by 1,10 phenanthroline was found to be competitive. One binding site was deduced on each isoenzyme for its corresponding inhibitor.
[Mahmoud A. Ibrahim, Abdel-Hady M.Ghazy, Mohamed N. Mosaad and Doaa A. Darwish. Purification and properties of alanine aminopeptidase from water buffalo kidney. Journal of American Science 2010;6(12):1600-1613]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Alanine aminopeptidase; water buffalo; kidney

1. Introduction

Aminopeptidases are ubiquitously distributed in animals, plants, bacteria as well as in fungi and catalyze the sequential removal of amino acid residues from the amino termini of peptides, polypeptides and proteins (Matsui et al., 2006; Bogra et al., 2009; Mane et al., 2010; Renwrantz and Lam, 2010).

Aminopeptidases are also vital for metabolic pathway regulation, cell maturation and turnover of proteins, including utilization of exogenous proteins as nutrient substances and elimination of non-functional proteins (Liu et al., 2008).

Aminopeptidase N (APN; EC 3.4.11.2), also called alanyl aminopeptidase (AAP) that cleaves neutral amino acids from the N-terminus of oligopeptides (Gabrilovac et al., 2005).

Also AAP are widely distributed in mammalian tissues and body fluids such as human seminal plasma (Huang et al., 1997), human liver (Yamamoto et al., 2000), rat liver (Yamamoto et al., 1998), human placenta (Mizutani et al., 1993), human pancreas (Sidorowicz et al., 1980), human kidney (Mantle et al., 1990), porcine kidney (Itoh and Nagamatsu, 1995), human gallbladder bile (Offner et al., 1994) and human skeletal muscle (Mantle et al., 1983). These enzymes from mammals are considered to participate in the metabolism of hormones and neurotransmitters (Hiroi et al., 1992).

AAP is an enzyme that is used as a biomarker to detect damage to the kidneys, and that may be used to help diagnose certain kidney disorders. It is found at high levels in the urine when there are kidney problems (Flynn, 1990).

Aminopeptidases participate in the development of flavour in food products, either directly, by hydrolyzing bitter peptides which are generally rich in hydrophobic amino acids and therefore good substrates for its action, or indirectly, where aminopeptidases could be involved in the liberation of aromatic amino acids which are important precursors of aroma compounds identified in cheese (Martinez-Cuesta et al., 2001).

The main goal of our research project is the production of industrial enzymes from the locally available rich sources. In the case of such enzyme (AAP), there is a need to develop economical and large-scale production methods for use in food industries. This work is the first report describes a simple purification procedure and some properties of aminopeptidase from the water buffalo kidney as a safe mammalian locally available rich source.

2. Material and Methods

Kidney materials

Fresh kidneys from water buffalo Bubalus bubalis, were obtained fresh from a local slaughter-house. The cortex and medulla were separated.

Chemicals:

Ampholyte solution, pH 3.5-10, ampholyte solution, pH 3-7, DL-alanine *β*-naphthylamide,1,4 Dithiothreitol (DTT), *p*-Chloromercuribenzoate, *p*-Hydroxy-mercuribenzoate, 1,10 phenanthroline, Phenymethylsulfonyl-fluoride (PMSF), diethyl-aminoethyl-cellulose (DEAE-Cellulose), bestatin, puromycin, glutathione reduced form, N-Tosylamide- L-phenyl-alanine chloromethyl ketone (TPCK), *Na-p*-Tosyl-L-Iysine chloromethyl ketone (TLCK), Phenylmethylsulfonyl fluoride (PMSF), Pepstatin A, soyabean trypsin inhibitor and Sephacryl S-300 were purchased from Sigma Chemical Co. The other chemicals were of analytical grade.

Purification of the kidney AAP

1- Preparation of crude extract

All of the procedures were performed at 4˚C unless stated otherwise. Thirty gm of frozen kidney cortex or medulla were sliced and homogenized in omni-mixer (Sorvall DuPont Instruments), with two volumes (w/v) of 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF. After centrifugation at 12,000 xg for 30 min at 4˚C, the supernatant was saved and designated crude extract.

2- DEAE-cellulose column Chromatography

The crude extract was chromatographed on DEAE-cellulose column previously equilibrated with 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF. The proteins were eluted with stepwise NaCl gradient ranging from 0 to 1 M followed by 0.5 % Triton X-100 prepared in the equilibration buffer. Fractions of 5 ml were collected at a flow rate of 60 ml / h and the fractions of the peaks containing the alanine aminopeptidase activity were pooled.

3- Sephacryl S-300 column Chromatography

The concentrated material containing the AAP activity was applied onto a Sephacryl S-300 column (142 cm x 1.75 cm i.d.). The column was equilibrated and developed with 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF at a flow rate of 30 ml / h. 2 ml fractions were collected. The Sephacryl S-300 column was used for molecular weight determination of the buffalo kidney cortex native AAP according to the method of Andrews, (1964 and 1965). The above described Sephacryl S-300 column was calibrated with ferritin (440 kDa), catalase (240 kDa), *β*-amylase (200 kDa) alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), myoglobin (17.2 kDa), and cytochrome C (12.4 kDa).

Assay of aminopeptidase activity

(A) Using *β*-naphthylamide derivatives

The aminopeptidase activity was assayed according to Kawata et al., (1980) by measuring the liberated *β*-naphthylamine. The reaction mixture 1.5 ml contained; 100 mM Tris-HCl buffer pH 7.8, suitable dilution of the enzyme extract and 0.4 mM DL-alanine-*β*-naphthylamide HCl. The reaction was initiated by adding 0.1 ml of the substrate (6 mM stock solution). The reaction was terminated by the addition of 0.5 ml of Fast Garnet GBC solution (1 mg / ml) in 1 M Na-acetate buffer pH 4.2 containing 10% Tween 20. The absorbance of the liberated *β*-naphthylamine was determined spectrophoto-metrically at 525 nm. One unit of AAP activity was defined as the amount of the enzyme which catalyzes the liberation of 1 nmol of *β*-naphthylamine per hour at 37˚C. The specific activity is expressed in units / mg protein. The *β*-naphthylamine concentration was determined from a previously constructed curve for *β*-naphthylamine treated similarly.

(B) By using *p*-nitroanilide derivatives

The aminopeptidase activity was determined according to Niven, (1995) by measuring the liberated *p*-nitroaniline in 1 ml reaction mixture containing 100 mM Tris-HCl buffer pH 7.8 and suitable dilution of the enzyme extract. The reaction was initiated by addition of substrate; 5 mM amino acyl *p*-nitroanilide derivatives dissolved in dimethylsulfoxide (DMSO). The reaction was terminated by the addition of 0.5 ml 30 % (v / v) acetic acid and the mixtures were centrifuged for 10 min at 10 000 xg. The absorbance at 405 nm was recorded against control lacking the enzyme and the *p*-nitroaniline concentration was determined from a previously constructed standard curve for *p*-nitroaniline treated similarly.

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% polyacrylamide gel according to Smith, (1969). SDS-PAGE was performed with 12% polyacrylamide according to Laemmli, (1970). The molecular weights of the purified AAP subunits were determined by SDS-PAGE as described by Weber and Osborn, (1969). The isoelectric point (pI) of the purified AAP was analysed on native 5% polyacrylamide vertical slabs (Robertson et al., 1987). The proteins were stained with 0.25% coomassie brilliant blue R-250.

Isoelectrofocusing marker proteins and their pI values; trypsinogen (9.3), lectin 1 (8.8), lectin 2 (8.6) lectin 3 (8.2), myoglobin 1 (7.2), myoglobin 2 (6.8), carbonic anhydrase 1 (6.6), carbonic anhydrase 2 (5.9), *β*-lactoglobulin (5.1), trypsin inhibitor (4.6)

and amyloglucosidase (3.6) were used to construct a calibration curve by plotting the distance from anode of each marker protein versus its isoelectric point pI value (Ubuka et al., 1987).

Staining of the AAP activity

Detection of AAP activity on gel was performed as described by Chien et al. (2002). After the native PAGE, the gel was incubated in the staining solution; 0.1 M Na-phosphate buffer pH 5.8 containing 1 mM $CoCl_2$, 0.06% DL-Alanine β-naphthylamide HCl and 0.06% Fast Garnet GBC until the development of the red bands. The stained gel was washed with water and then fixed in 7% acetic acid.

Protein determination

Protein was determined by the dye binding assay method (Bradford, 1976). Bovine serum albumin was used as a standard protein.

3. Results

Comparison of the AAP activity in the kidney cortex and medulla:

The AAP activity was assayed and compared in the kidney cortex and medulla. The AAP showed a higher specific activity in the cortex (1616.13 ± 91.77 units / mg protein) than the medulla (194.4 ± 10.7 units / mg protein) representing more than 8 folds Table (1). Therefore, the kidney cortex is selected for the AAP purification.

Table (1), AAP specific activity in the water buffalo kidney cortex and medulla.

Specific activity* of the water buffalo kidney AAP by using DL-alanine β-naphthylamide	
Cortex	Medulla
1616.1 ± 91.8	194.4 ± 10.7

Purification of AAP from the water buffalo kidney cortex

A typical purification scheme of the AAP from water buffalo kidney cortex is presented in Table (2). The procedure involved anion exchange chromatography on DEAE-cellulose column (Fig. 1) followed by gel filtration chromatography on Sephacryl S-300 column (Fig. 2 A, B and C). The starting specific activity in the crude extract was 1704.25 unit / mg protein. The chromatography on the DEAE-cellulose column revealed the presence of three peaks of the AAP activity eluted with 0.1 M NaCl (AAP1), 0.2 M NaCl (AAP2) and 0.5% Triton X-l00 (AAP3) (Fig. 1).

For further purification, the concentrated pooled fractions of AAP1, AAP2 and AAP3 were applied separately on a Sephacryl S-300 column. The column was equilibrated and developed with 0.02 M Tris-HCl buffer pH 7.8 containing 1 mM $MgCl_2$ and 0.2 mM PMSF. The elution profiles of AAP1, AAP2 and AAP3 (Fig. 2 A, B and C) revealed the presence of one peak of AAP activity in each one. After the gel filtration on the Sephacryl S-300 column the specific activity of AAP1, AAP2 and AAP3 were increased to 2972.1, 4038.06 and 11655.32 units / mg protein which represents 1.74, 2.36 and 6.83 fold purification over the crude extract with 5.18 %, 8.6 % and 51.31 % recovery respectively.

The molecular weights of the native AAPs were calculated from the calibration curve to be 120 kDa, 400 kDa and 310 kDa for AAP1, AAP2 and AAP3 respectively. The elution volumes (Ve) of AAP1, AAP2 and AAP3 are 190 ml, 140 ml and 146 ml respectively from the Sephacryl S-300 column (Fig. 2A, 2B and 2C) and the column void volume (Vo) was 132 ml as determined by the dextran blue (2000 kDa).

Characterization of the water buffalo kidney cortex AAPs

Electrophoretic analyses

` Samples from the different purification steps (crude extract, DEAE-cellulose and Sephacryl S-300 fractions) of AAP1, AAP2 and AAP3 were analyzed electrophoretically on native 7% polyacrylamide gel (Fig. 3). The isoenzyme pattern of the crude extract and the three isoenzymes confirmed that the water buffalo kidney cortex contain three distinct AAP isoenzymes. The AAP activity was visualized on 7% native PAGE. AAP1 and AAP2 isoenzymes migrated faster than AAP3 due to their higher negative charge (Fig. 4).

The native and denatured 12% SDS-PAGE confirmed the purity of water buffalo kidney cortex AAP1, AAP2 and AAP3 (Fig. 6). The subunit molecular weights of AAP1, AAP2 and AAP3 were estimated to be 60 ± 1 kDa, 67 ± 1 kDa and 53 ± 1 kDa respectively (Fig. 6).

Samples of the purified AAP1, AAP2 and AAP3 isoenzymes were electrofocused (Fig. 5). AAP1, AAP2 and AAP3 isoenzymes showed isoelectric points (p*I*) value at pH 5.6, 4.9 and 4.6 respectively.

Substrate specificity

The substrate specificity of the purified water buffalo kidney AAP1, AAP2 and AAP3 were screened toward various substrates and presented in (Table 3). The three isoenzymes AAP1, AAP2 and AAP3 cleaved preferentially alanyl residue (100 % relative activity).

The rate of hydrolysis of DL-alanine -*β*-naphthylamide HCl (0.311), (0.327) and (0.827) were more than the rate of hydrolysis of L- alanine *p*-nitroanilide (0.231), (0.244) and (0.763) for AAP1, AAP2 and AAP3 respectively.

Effect of pH on AAP1, AAP2 and AAP3

The enzyme activity of AAP1, AAP2 and AAP3 isoenzymes toward DL-alanine-*β*-naphthylamide HCl were measured in 100 mM Tris-HCl buffer of various pH values from 7.2 to 9. The optimum pH was found to be at pH 8, 7.8 and 7.8 for AAP1, AAP2 and AAP3 respectively (Fig. 7).

Table (2). A typical purification scheme of the water buffalo kidney cortex AAP.

Purification step	Total protein (mg)	Total units	Recovery (%)	Specific activity	Fold purification
Crude extract	400	681700.6	100.0	1704.3	1.0
DEAE-cellulose fractions					
0.1 M NaCl (AAP1)	61.6	62714.5	9.1	1017.6	0.6
0.2 M NaCl (AAP2)	70.4	70444.5	10.3	1000.2	0.6
Triton X-l00 (AAP3)	107.3	466587.4	68.4	4346.1	2.6
Sephacryl S-300					
0.1 M NaCl (AAP1)	11.9	35349.0	5.2	2972.1	1.7
0.2 M NaCl (AAP2)	14.5	58632.1	8.6	4038.1	2.4
Triton X-l00 (AAP3)	30.0	349793.1	51.3	11655.3	6.8

1- All data based on 15 gm water buffalo kidney cortex.
2- One unit of alanine aminopeptidase activity was defined as the amount of the enzyme which catalyzes the liberation of 1 nmol of *β*-naphthylamine per hour at 37°c.
3- The specific activity is expressed as units / mg protein.

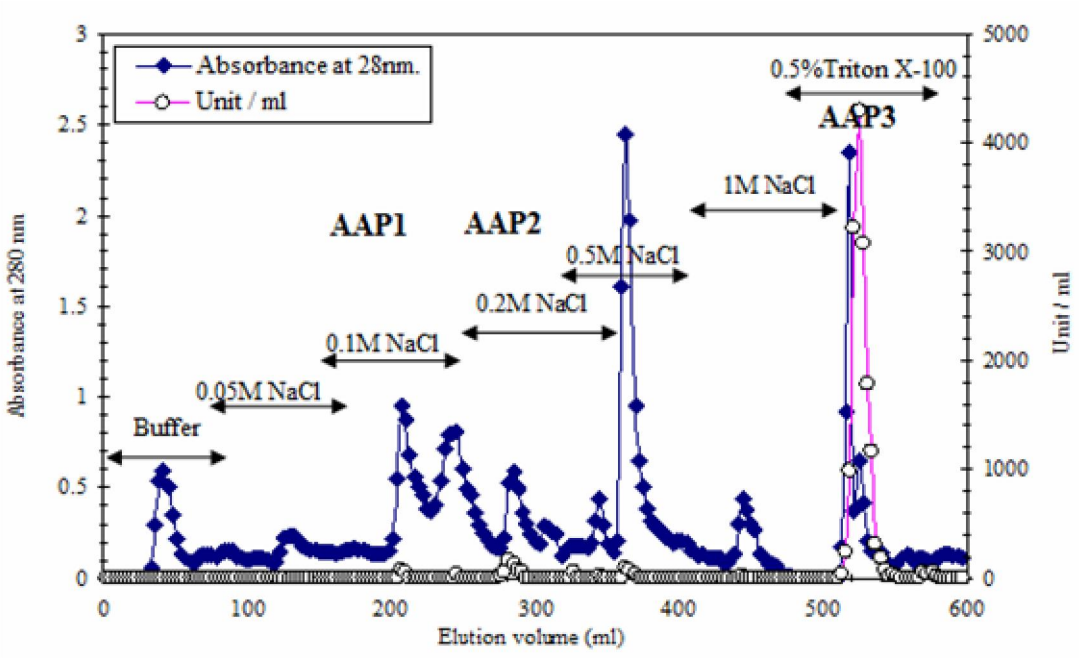

Figure 1. A typical elution profile for the chromatography of the water buffalo kidney cortex crude extract on DEAE-cellulose column (43 cm x 2.6 cm i.d.).

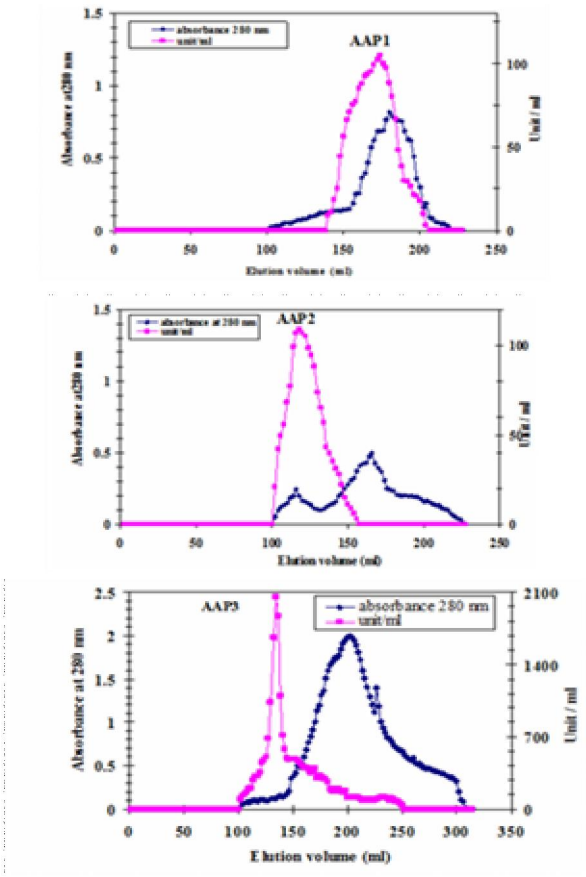

Figure 2. A typical elution profiles for the chromatography of concentrated pooled DEAE-cellulose fractions AAP1, AAP2 and AAP3 on Sephacryl S-300 column (142 cm x 1.75 cm i.d.).

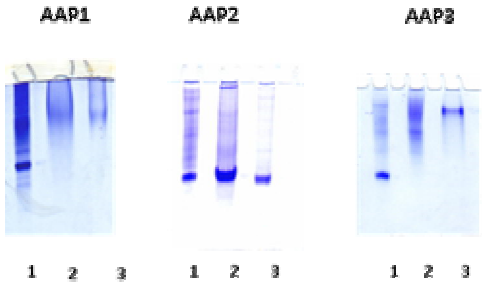

Figure 3. Electrophoretic analysis of protein pattern of the different purification steps of AAP on 7% native polyacrylamide gel: (1) water buffalo kidney cortex crude extract, (2) concentrated DEAE-cellulose fraction and (3) Sephacryl S-300 purified fraction.

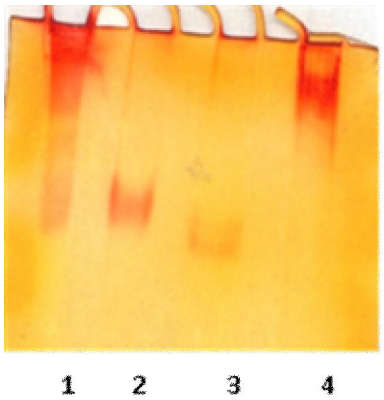

Figure 4. Isoenzyme pattern on 7% native polyacrylamide gel: (1) crude extract, (2) AAP1, (3) AAP2 and (4) AAP3.

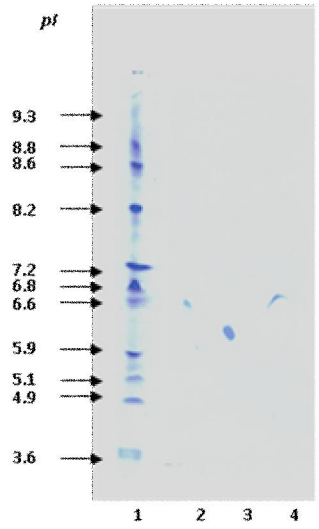

Figure 5. Isoelectrofocusing; [1] Isoelectric point (*pI*) marker proteins, [2] AAP1, [3] AAP2 and [4] AAP3.

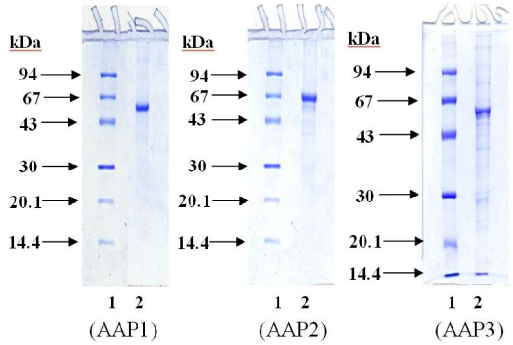

Figure 6. 12% SDS-polyacrylamide gel electrophoresis of (1) low molecular weight marker proteins (2) denatured purified water buffalo kidney cortex AAP.

Table (3), Substrate specificity of the water buffalo kidney cortex AAP1, AAP2 and AAP3.

Substrate	Concentration	AAP1		AAP2		AAP3	
		Rate of hydrolysis	Relative activity	Rate of hydrolysis	Relative activity	Rate of hydrolysis	Relative activity
L-Alanine *p*-nitroanilide HCI	1mM	0.231	100.0 %	0.244	100.0 %	0.763	100.0 %
L-Leucine *p*-nitroanilide	1mM	0.129	55.8 %	0.208	85.2 %	0.689	90.3 %
Glycine *p*-nitroanilide	1mM	0.046	19.9 %	0.06	24.6 %	0.192	25.2 %
S-Benzyl-L-Cysteine *p*-nitroanilide	1mM	0.134	13.4 %	0.052	21.3 %	0.171	22.4 %
L-Lysine *p*-nitroanilide 2HBr	1mM	0.079	34.2 %	0.111	45.4 %	0.252	33.0 %
L-Arginine *p*-nitroanilide 2HCI	1mM	0.068	29.4 %	0.097	39.7 %	0.325	42.6 %
L-Valine-*p*-nitroanilide HCl	1mM	0.011	4.7 %	0.024	9.8 %	0.15	19.7 %
L-Phenylalanine *p*-nitroanilide HCI	1mM	0.037	16.0 %	0.083	34.0 %	0.316	41.4 %
L-Glutamic acid y-(*p*-nitroanilide)	1mM	0.164	70.9 %	0.183	75.0 %	0.616	80.7 %
L-Proline *p*-nitroanilide	1mM	0.031	13.4 %	0.04	16.4 %	0.163	21.4 %
DL-Alanine *β*-naphthylamide HCI	0.4mM	0.311	100.0 %	0.327	100.0 %	0.827	100.0 %
Glycine *β*-naphthylamide HCI	0.4mM	0.078	25.0 %	0.052	15.9 %	0.099	12.0 %
L-Leucine *β*-naphthylamide HCI	0.4mM	0.206	66.0 %	0.229	70.0 %	0.569	68.8 %

Michaelis-Menten constant (Km) value

The purified AAP1, AAP2 and AAP3 isoenzymes were incubated with increasing concentrations of DL- alanine -*β*-naphthylamide HCl. The plots of substrate concentration [s] versus reaction velocity (v) (Fig. 8) were used to calculate the Michaelis-Menten constants (Km). The Km values were found to be 0.15, 0.17 and 0.125 mM and the corresponding maximum velocities (Vmax) were calculated to be 1694, 1143 and 66129 units / mg protein for AAP1, AAP2 and AAP3 respectively.

Effect of divalent cations on AAP1, AAP2 and AAP3

The purified AAP1, AAP2 and AAP3 isoenzymes were pre-incubated with 0.5 and 1.0 mM of each divalent cation at 37ºC and the activity was assayed. Table (4) shows the activity of AAP1, AAP2 and AAP3 in the presence of each cation. A control test based on the rate of hydrolysis of DL-alanine-*β*-naphthylamide HCl without any cation was taken as 100 % relative activity.

The activity of AAP3 was increased 112.5 % and 108.2 % in the presence of 0.5 and 1.0 mM $MgCl_2$ and 107.9 % and 100.8 % in the presence of 0.5 and 1.0 mM $CaCl_2$ respectively. The three isoenzymes were inhibited by the metal ions of Cu^{2+}, Mn^{2+} and Ni^{2+}, while Co^{2+} and Fe^{2+} have non-significant effect on AAP3 but have inhibitory effects on AAP1 and AAP2. The metal ion Zn^{2+} has a great inhibitory effect on the three isoenzymes AAP1, AAP2 and AAP3.

Effect of amino acids on AAP1, AAP2 and AAP3

Prior to the reaction initiation with the substrate, 1 mM of each tested amino acid was incubated with the enzyme in the assay reaction mixture. Table (5) presents the effect of the different amino acids on the purified water buffalo kidney cortex AAP1, AAP2 and AAP3 isoenzymes.

Almost all amino acids increase the activity of AAP2 except L- tyrosine, phenylalnine and L-leucine caused either slight or moderate inhibition of the enzyme. The isoenzymes AAP1 and AAP3 were inhibited 47.4 % and 32.9 % in the presence of 1 mM of tyrosine, 30 and 17.8 % by 1 mM phenylalnine and 25 % and 7.7 % by 1 mM serine respectively. The other tested amino acids caused slight inhibition of the isoenzymes AAP1 and AAP3.

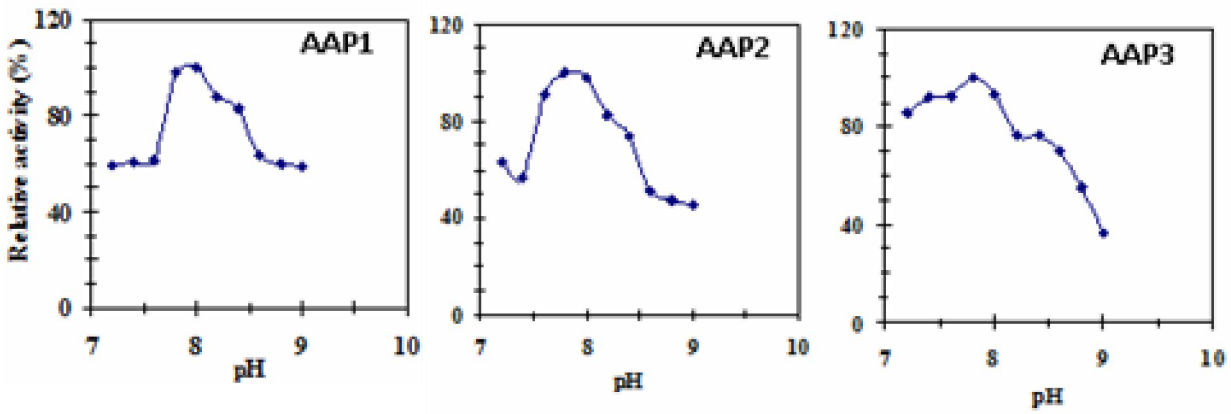

Figure 7. Effect of pH on the purified water buffalo kidney cortex AAP1, AAP2 and AAP3 using DL-alanine-*β*-naphthylamide HCl as substrate in 100 mM Tris-HCl buffer of various pH values from 7 to 9.

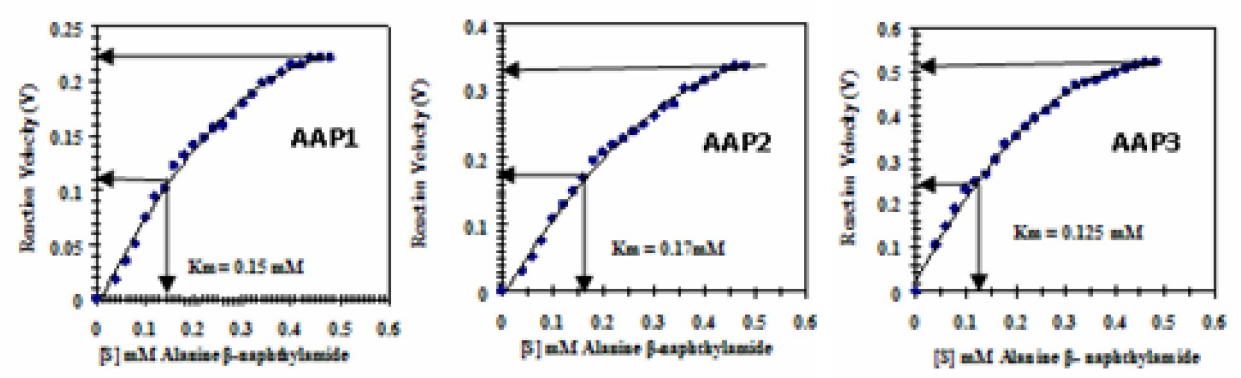

Figure 8. Effect of the substrate DL-alanine-β-naphthylamide HCl concentration in mM on the reaction velocity of the purified water buffalo kidney cortex AAP1, AAP2 and AAP3. The reaction velocity is the change in absorbance at 525nm per 30 min.

Effect of inhibitors on AAP1, AAP2 and AAP3

The purified AAP1, AAP2 and AAP3 isoenzymes were pre-incubated with a suitable concentration of each inhibitor at 37ºC for 5 min and the residual activity was assayed (Table 6).

The DL-dithiothreitol (DTT) was an obvious inhibitor of AAP1 since it caused 97.9 % inhibition at a concentration of 0.4 mM, while 1,10 phenanthroline was an inhibitor of both AAP2 and AAP3 since it caused 94.4 % and 96.5 % inhibition at a concentration of 10 mM.

The bestatin was a potent inhibitor of AAP1, AAP2 and AAP3 since it caused 72 %, 82.1 % and 79.56 % inhibition respectively at a concentration of 1 µM.

The isoenzymes AAP2 and AAP3 were sensitive to the thiol compound *β*-mercaptoethanol since it inhibited 60.13 % and 58.6 % of the enzyme activity at a concentration of 0.4 mM and also they were sensitive to reduced glutathione (GSH), which inhibited 57.69 % and 36.18 % of the enzyme activity respectively.

Kinetics of AAP1, AAP2 and AAP3 inhibition by DTT and 1,10 Phenanthroline

The effect of varying concentrations of DTT on the AAP1 activity is shown in Fig. (9). A maximum inhibition of AAP1 by DTT 92 % was reached at a concentration of 0.4 mM. A linear relationship was observed by constructing the Hill

plot for the inhibition of the purified water buffalo kidney cortex AAP1 by DTT, a straight line was obtained with slope of about 1.1 for AAP1 Fig. (9). Also, the effect of varying concentrations of 1,10-phenanthroline on the AAP2 and AAP3 activity are shown in (Fig. 10 and 11). A maximum inhibition of AAP2 and AAP3 by 1,10 phenanthroline 94.1 % and 91.2 % was reached at 10 mM 1,10-phenanthroline. The slopes of the Hill plot were found to be 0.95 and 0.78 for AAP2 and AAP3 (Fig. 10 and 11)**.**

Table (4**),** Effect of divalent cations on water buffalo kidney cortex AAP1, AAP2 and AAP3.

Reagent	**Final concentration (mM)**	**Residual activity (%)**		
		AAP1	**AAP2**	**AAP3**
Control	-	100.0	100.0	100.0
$CuCl_2$	0.5	64.7	87.6	74.1
	1.0	60.6	80.3	67.2
$MnCl_2$	0.5	45.5	84.3	88.9
	1.0	34.7	71.3	85.2
$ZnCl_2$	0.5	17.6	19.7	1.7
	1.0	15.0	11.8	0.0
$NiCl_2$	0.5	43.5	48.9	55.6
	1.0	37.3	34.3	40.0
$CoCl_2$	0.5	70.9	73.0	100.0
	1.0	65.2	75.2	99.1
$CaCl_2$	0.5	64.2	69.1	107.9
	1.0	61.6	67.4	100.8
$FeCl_2$	0.5	89.6	83.7	99.1
	1.0	86.5	86.5	95.6
$MgCl_2$	0.5	92.2	84.8	112.5
	1.0	86.5	87.6	108.2

The inhibition of AAP1, AAP2 and AAP3 by DTT and 1,10 phenanthroline were competitive type since the presence of the inhibitors did not alter the Vmax value but increases the Km value (Fig. 9, 10 and11).

The Ki value of AAP1 inhibition by DTT is determined to be 47 µM directly from the intercept of the X axis of the plot, while the Ki values of AAP2 and AAP3 inhibition by 1,10 phenanthroline are determined to be 1.3 mM and 1.9 mM respectively (Fig. 9, 10 and 11).

Table (5), Effect of amino acids (1 mM final concentration) on water buffalo kidney cortex AAP1, AAP2 and AAP3.

Amino acid	**Residual activity (%)**		
	AAP1	**AAP2**	**AAP3**
Control	100.0	100.0	100.0
L-Alanine	93.5	127.3	99.2
L-Tyrosine	67.1	83.1	52.6
DL-Phenylalnine	82.1	80.4	69.8
DL-Tryptophane	73.1	114.5	86.4
L-Lysine	90.4	118.0	98.7
L-Methionine	95.9	125.0	99.3
L-Histidine	90.4	120.0	93.2
L-Leucine	84.5	85.7	83.4
L-Arginine	86.8	121.9	98.3
L-Glutamine	90.4	123.8	97.7
L-Serine	92.2	130.0	74.9

4. Discussion

Among various aminopeptidases, the alanyl aminopeptidases (AAPs) which preferentially liberate amino-terminal neutral amino acids, such as Ala, Met, Leu and Tyr of peptides are widely distributed in mammalian tissues and body fluids (Mane et al., 2010).

In this study, a simple, convenient and reproducible purification procedure of the water buffalo kidney cortex AAP is carried out by a combination of anion exchange chromatography on DEAE-cellulose column followed by gel filtration chromatography on Sephacryl S-300 column. The native enzyme was obtained since both the extraction buffer and the equilibration buffer of the DEAE-cellulose column contained 0.2 mM phenylmethylsulfonylfluoride (PMSF), a serine protease inhibitor to avoid the action of the endogenous protease.

On the basis of the protein charge, the chromatography on DEAE-cellulose column revealed the presence of three forms of water buffalo kidney

cortex alanine aminopeptidases AAP1, AAP2 and AAP3 (Fig. 1). A considerable yield and purity of the AAP isoenzymes from water buffalo kidney cortex was obtained (Table 2). The recovery % of the enzyme units was 9.1, 10.3 and 68.44 for AAP1, AAP2 and AAP3 respectively. The total recovery is more than 87%. The alanine aminopeptidase from chicken intestine has a yield of 3.43% (Mane et al., 2010).

Table (6), Effect of inhibitors on the purified water buffalo kidney AAP1, AAP2 and AAP3.

Inhibitor	**Final concentration**	**Inhibition %**		
		AAP1	**AAP2**	**AAP3**
Control		0.0	0.0	0.0
Bestatin HCI	1.0 µM	72.0	82.1	79.6
Puromycin	1.0 mM	30.7	6.6	27.4
p-Chloromercuribenzoic acid (*p*CMB)	0.2 mM	0.0	5.9	29.3
p-Hydroxymercuribenzoate (*p*HMB)	0.2 mM	33.6	34.6	14.7
β - Mercaptoethanol	0.4 mM	46.0	60.1	58.6
DL-Dithiothreitol (DTT)	0.4 mM	97.9	88.5	73.9
L-Cysteine	1.0 mM	0.0	19.2	13.3
Glutathione reduced form (GSH)	1.0 mM	22.0	57.7	36.2
N-Ethylmaleimide	1.0 mM	0.0	15.0	2.7
N-Tosylamide-L-phenylalanine chloromethyl ketone (TPCK)	1.0 mM	0.0	30.4	15.0
EDTA	1.0 mM	0.0	3.8	0.0
Na-p-Tosyl-L-Iysine chloromethyl ketone (TLCK)	1.0 mM	0.0	39.5	2.0
Phenylmethylsulfonylfluoride (PMSF)	1.0 mM	7.6	15.0	12.5
1,10 Phenanthroline	10 mM	91.0	94.4	96.5
Pepstatin A	10 mM	0.0	0.0	0.0
Iodoacetic acid	10 mM	0.0	0.0	0.0
Soya bean trypsin inhibitor	15 µg	0.0	0.0	0.0

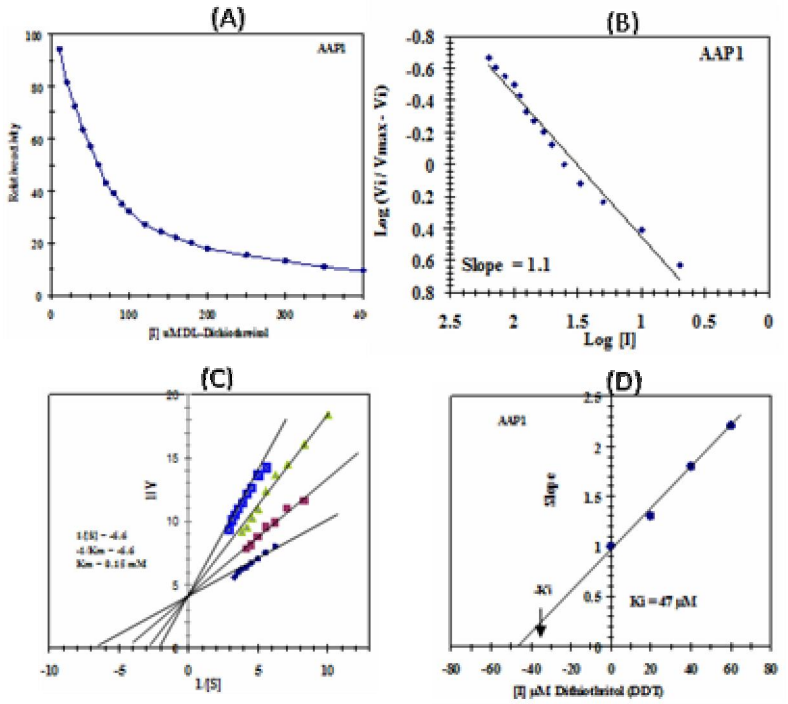

Figure 9. (A) Inhibition AAP1 by varying concentrations of DTT. (B): Hill plot for inhibition of AAP1 by varying concentrations of DTT. (C): Lineweaver-Burk plots showing the type of inhibition of AAP1 by DTT. (D): Determination of the inhibition constant (Ki) value for the inhibition of t AAP1 by DTT.

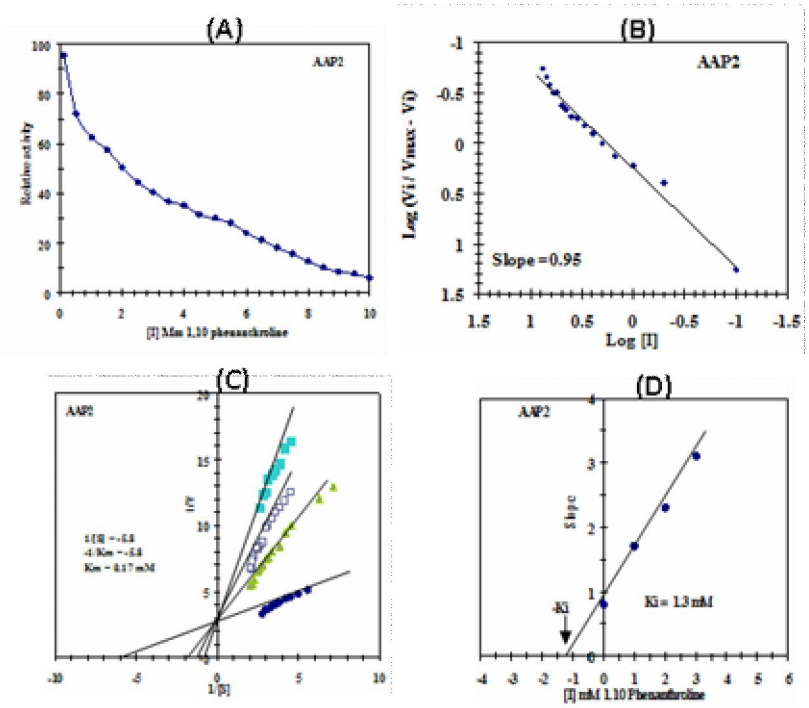

Figure 10. (A) Inhibition of the purified AAP2 by varying concentrations of 1,10-phenanthroline. (B): Hill plot for inhibition of AAP2 by varying concentrations of 1,10-phenanthroline. (C): Lineweaver-Burk plots showing the type of inhibition of AAP2 by 1,10-phenanthroline. (D): Determination of the inhibition constant (Ki) value for the inhibition of AAP2 by 1,10-phenanthroline.

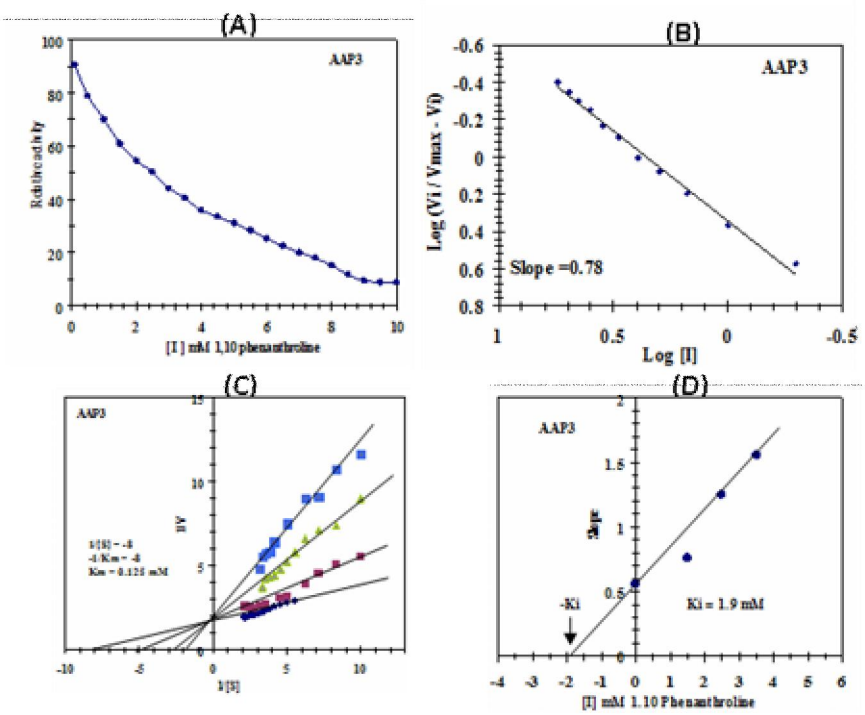

Figure 11. (A) Inhibition of the purified AAP3 by varying concentrations of 1,10-phenanthroline. (B): Hill plot for inhibition of AAP3 by varying concentrations of 1,10-phenanthroline. (C): Lineweaver-Burk plots showing the type of inhibition of AAP3 by 1,10-phenanthroline. (D): Determination of the inhibition constant (Ki) value for the inhibition of AAP3 by 1,10-phenanthroline.

The molecular weights of the native isoenzymes were determined by gel filtration to be 120 kDa, 400 kDa and 310 kDa for AAP1, AAP2 and AAP3 respectively (Fig. 2). The molecular mass of AAP1 was different from those of 180 kDa from chicken intestine alanine aminopeptidase (Mane et al., 2010), 153 kDa from human seminal plasma (Huang et al., 1997), 140 kDa from rat kidney (Watt et al., 1989), 106 kDa from porcine skeletal muscle (Flores *et al.*, 1996), 102 kDa from human skeletal muscle (Mantle et al., 1983) and 98 kDa from human liver cytosol (Yamamoto et al., 2000). The molecular masses of AAP2 and AAP3 were more or less similar to those of 420 kDa from gypsy moth *Lymantria dispar* (Masler and Kovaleva, 1997), 375 kDa from eosinophilic blood cells of *Mytilus edulis* (Renwrantz and Lam, 2010), 320 kDa from human placenta (Lampelo et al., 1982), 290 kDa from ostrich duodenal mucosa (Roos et al., 1993), 280 kDa from pig kidney (Wacker, 1976) and 236 kDa from human kidney (Kao et al., 1978).

All of the AAP1, AAP2 and AAP3 turned out to be homogenous as indicated by both native (Fig. 3) and SDS-PAGE (Fig. 6). Also, the single band of the enzyme subunit (Fig. 5) confirms the purity of the enzyme.

The subunits molecular weight of the purified buffalo kidney cortex AAP1 is determined by SDS-PAGE to be 60 ± 1 kDa indicating a homodimeric structure composed of two identical subunits, while AAP2 was 67 ± 1 kDa indicating a homohexameric structure composed of six identical subunits and AAP3 was 53 ± 1 kDa suggesting also a homohexameric structure composed of six identical subunits. (Fig. 6). However, aminopeptidases exhibit molecular weights ranging from 53–140 kDa per subunits and exist as monomers, hexamers and octamers (Božić et al., 2008).

The estimated p*I* values at pH 6.4, 6.2 and 6.6 for AAP1, AAP2 and AAP3 respectively (Fig. 5) are slightly higher than that described previously. The isoelectric point (pI) value is found to be 5.0 for membrane alanyl aminopeptidase (Riemann et al., 1999), between pH 4.5 and 5.8 for the soluble and surface-bound aminopeptidase in *Mytilus edulis* blood cells (Renwrantz and Lam, 2010) and 4.9 for that from rat liver (Hiroi et al., 1992).

The isoenzymes AAP1, AAP2 and AAP3 cleaved preferentially alanyl residue (Table 3)

The Km values using alanine *β*-naphthylamide as substrate were found to be 0.15, 0.17 and 0.125 mM for AAP1, AAP2 and AAP3 respectively (Fig. 8). These values were considerably

close to alanyl aminopeptidase from chicken intestine, since Km value of preferentially hydrolyzed Leu-β-NA was found to be 0.1 mM (Mane et al., 2010) indicating the high affinity of the buffalo kidney cortex AAPs toward the alanine β-naphthylamide.

The water buffalo kidney cortex AAP1, AAP2 and AAP3 displayed their optimum activity at pH 8, 7.8 and 7.8 respectively (Fig. 7). These pH values are similar to that of monomeric alanine aminopeptidase from bovine skeletal muscle which was optimal at pH 8.0 (Ye and Ng, 2011) and pH 7.9 for human pancreas alanine aminopeptidase (Sidorowicz et al., 1981).

The activity of AAP3 was increased in the presence of $MgCl_2$ and $CaCl_2$. The metal ion Zn^{2+} has a great inhibitory effect on the three isoenzymes AAP1, AAP2 and AAP3 (Table 4). High concentrations of Zn^{2+} (in the mM range) often inhibit metallopeptidases due to the formation of zinc monohydroxide that bridges the catalytic Zn^{2+} ion to a side chain in the active site of the enzyme (Božić et al., 2008). The chicken intestinal alanine aminopeptidase show that presence of cations (Zn^{2+} and Mn^{2+}) slightly activated the enzyme activity (Mane et al., 2010). The bovine skeletal muscle monomeric alanine aminopeptidase activity was totally abolished by Co^{2+} and Zn^{2+} ions, and almost completely inhibited by Mn^{2+}, the activity was strongly inactivated by Mg^{2+}, and Fe^{3+} ions. However the activity was not affected by Ca^{2+} (Ye and Ng, 2011).

The susceptibility of aminopeptidases to inhibition by free amino acids, suggesting in turn that the degradation of oligopeptides to amino acids may play a key role in control of the overall protein turnover process **(**Toldra et al., 1996). It has been suggested that the levels of the free amino acids within the cell may regulate the protein synthesis/degradation cycle, via a feedback inhibition type **(**Mader, 1988**)**; because aminopeptidase enzymes are in turn subject to inhibition by free amino acids **(**McDonald and Barrett, 1986**)**, it follows that the ultimate function of soluble aminopeptidases may be involved in the control of cellular protein turnover **(**Mantle, 1992**)**. Therefore, the effect of amino acids on the three isoenzymes AAP1, AAP2 and AAP3 was studied and presented in Table (5). The tyrosin inhibited the three isoenzymes obviously.

In the present study, the effect of different specific and characteristic inhibitors on the water buffalo kidney cortex AAPs is presented in Table (6). The purified isoenzymes are resistant to the serine protease inhibitors PMSF, N-Tosylamide-L-phenylalanine chloromethyl ketone (TPCK), Nα-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK) and soya bean trypsin inhibitor indicating that the enzyme active site does not contain a serine residue and this was the reason why PMSF was added to the tissue homogenization buffer to inhibit the endogenous serine proteases. Also, they do not belong to the acid or thiol proteases groups since both the acid protease inhibitor, pepstatin A and thiol protease inhibitor, iodoacetic acid did not affect the purified AAP isoenzymes. The three isoenzymes AAP1, AAP2 and AAP3 are not cysteine proteases due to their resistance to the cysteinyl protease inhibitors, *p*-chloromercuribenzoic acid (*p*CMB), *p*-hydroxymercuribenzoate (*p*HMB) and N-ethylmaleimide (Table 6). Lack of enzyme inhibition with cysteine and serine protease inhibitors suggests that purified aminopeptidase does not have any endopeptidase activity (Pokharel and Rathaur, 2008).

The inhibition of the AAP isoenzymes by the thiol compound DTT indicates the role of sulfhydryl group in enzyme catalysis and their inhibition by 1,10 phenanthroline indicates that the isoenzymes are metalloenzymes.

Similarly, aminopeptidases from porcine liver (Imamura et al., 1983) and rabbit kidney (Oliveira et al., 1999) are found to be metalloenzymes. Inhibition by chelating agents such as 1,10-phenanthroline indicates the presence of at least one divalent zinc cation associated with the enzyme active site and should be considered as a zinc-aminopeptidase (Pokharel and Rathaur, 2008). Bestatin is not only LAP inhibitor but also a well-recognized inhibitor of membrane alanyl aminopeptidase although considerably less potent than amastatin or probestin **(**Tieku and Hooper, 1992). In this study, bestatin was found to be a potent inhibitor of the three AAP isoenzymes (Table 6) confirming that all of them are alanine aminopeptidase.

From the titration curves data a linear relationship was observed by constructing the Hill plot for the inhibition of the purified AAP1 by DTT and AAP2 and AAP3 by 1, 10 phenanthroline. The slope of the Hill plot was found to be 1.1 for AAP1 whereas 0.95 and 0.78 for AAP2 and AAP3 indicating the existence of one binding site for DTT and 1, 10 phenanthroline (Fig. 9, 10 and 11).

The type of inhibition of AAP1 by DTT, and AAP2 and AAP3 by 1, 10 phenanthroline were found to be competitive where the presence of DTT and 1, 10 phenanthroline did not alter the Vmax value but increased the Km value. For the determination of the Ki value, the slopes of the reciprocal plots lines were plotted against the DDT and 1, 10 phenanthroline concentrations. The Ki value of the AAP1 inhibition by DTT was determined to be 47 µM, whereas the Ki values of AAP2 and AAP3 inhibition by 1, 10 phenanthroline were determined to be 1.3 mM and

1.9 mM respectively directly from the intercept of the X axis of the plot (Fig. 9, 10 and 11).

The hexameric structure of AAP2 (400 kDa) and AAP3 (310 kDa) and their similar catalytic properties suggest that AAP2 may be a precursor of AAP3 and proteolytic modification is involved in the conversion of AAP2 to AAP3. On the other hand, AAP1 could be considered a unique isoform.

In conclusion, this study presents a simple, convenient and reproducible method for the purification of a well characterized alanine aminopeptidases from the water buffalo kidney cortex as a safe locally available rich source. Production of these enzymes on large scale will allow their use in various applications such as food industries and also investigation of the protein primary structure.

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11/22/2010