J. Indian Chem. Soc.,
Vol. 80, November 2003, pp. 1019-1025

Purification and characterization of dipeptidylpeptidase IV from goat brain†

Ashwani Mittal[a], Shiwani Khurana[a], Rachna Sadana[a], Hari Singh[a]* and Ramesh C. Kamboj[b]

[a]Department of Biochemistry, Kurukshetra University, Kurukshetra-136 119, India

E-mail : singhharikuk@rediffmail.com *Fax* : 91-1744-238277

[b]Department of Chemistry, Kurukshetra University, Kurukshetra-136 119, India

Manuscript received 3 September 2003

Dipeptidylpeptidase IV (DPP IV) proteinase was purified ~133-fold with specific activity 29.2 units/mg to an apparent electrophoretic homogeneity. It was isolated from goat brain by homogenization, acid-autolysis at pH 6.0, 20–50% $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on CM-Sephadex C-50 at pH 5.87, Sephadex G-150 gel filtration and anion-exchange chromatography on DEAE-Sephadex A-50 at pH 8.0. The molecular weight of the enzyme estimated by gel-filtration chromatography was ~200000 Da. The pH optimum of the enzyme for the hydrolysis of Gly-Pro-4mβNA was 8.0. Of the synthetic chromogenic substrates tested, Ala-Pro-4mβNA was hydrolyzed maximally by DPP-IV enzyme followed by Gly-Pro-4mβNA. K_m value for the hydrolysis of Gly-Pro-4mβNA was 0.5 m*M*. The enzyme activity of the purified preparation was inhibited by PMSF, TLCK, puromycin and heavy metal ion i.e. Zn^{2+} but was not inhibited by *p*-hydroxy-mercuriphenylsulphonic acid, EDTA, iodoacetic acid, indicating that goat brain DPP IV is a serine protease. The dipeptides, His-Pro and Ala-Pro, competitively inhibited the hydrolysis of Gly-Pro-4mβNA. The enzyme was stable between pH 6.8–9.0 and upto 50°. The optimum temperature for the hydrolysis of Gly-Pro-4mβNA was ~50° with an activation energy E_a of ~11 kcal mol^{-1}. These properties are similar to those of DPP IV purified from other tissues. The purification and characterization of DPP IV is of great value for the identification of its natural substrates and for the study of its physiological significance in the brain tissue.

Membrane bound proteases are widely distributed among various cell systems. Their expression in a particular cell type is finely regulated, reflecting the specific functional cell implication and engagement in defined physiological pathways. The presence of proline residues gives unique structural features to peptide chain, subsequently influencing the susceptibility of proximal peptide bond to protease cleavage. Peptide bonds involving the cyclic amino acid proline not only influence the conformation of peptide chain but also restrict the attack by most proteases, even those with broad specificity. Among the rare group of proline specific proteases, dipeptidyl peptidase IV [EC 3.4.14.5] is one of the very few membrane bound enzyme specific for proline. This is an exopeptidase serine protease that preferentially liberates X-proline or sometimes X-alanine dipeptides from the N-termini of some polypeptides[1–3] at pH 7.8–8.7. This protease degrades[3–5] several biologically active peptides, cytokines and hormones such as substance P, interleukin-8 (IL-8), growth hormone-releasing hormone (GH-RH), gastrin releasing peptides and α-chain of human chorionic gonadotropin (HCG) which indicates its important role in regulatory processes. This protease is also involved in various diseases like cancer[6], systemic lupus erythematosus[7], rheumatoid arthritis[7,8], schizophrenia[9], type-2-diabetes[10], and in human immunodeficiency vrius (HIV) infection in $CD4^+$ cells[11]. Work dealing with mechanistic features of this proline specific enzyme, therefore, is of general interest. This protease was first identified in rat liver[1] by Hopsu-Havu and Glenner in 1966 and was subsequently purified from various mammalian tissues and body fluids such as kidney[12,13], liver[13–15], submaxillary gland[16], seminal plasma[17], serum[18] and cerebrospinal fluid[19]. DPP IV activity has also been reported[20–23] in insect, yeast, bacteria and plants. The literature survey has revealed that complete studies pertaining to this protease in brain have not been undertaken so far. This enzyme in the brain may cleave X-Pro dipeptide from some biologically active peptides such as substance P, neuropeptide Y, bradykinin etc., which act as neurotransmitters and modulator of the hormonal activities. These reasons prompted us to investigate and establish the presence of DPP IV enzyme in brain. In the present study, the enzyme has been purified by using a simple procedure and has been characterized in terms of its molecular weight, substrate specificity, kinetic parameters, pH and temperature

†Dedicated to Professor S. M. Mukherji, our revered teacher and Founder Head of Chemistry Department of Kurukshetra University, Kurukshetra.

optima, pH and temperature stability, activation and inhibition studies.

Results and discussion

Purification of DPP IV proteinase :

The procedure adopted for purification of DPP IV proteinase from goat brain is simple and rapid. It involves only three chromatographies i.e. cation-exchanger CM-Sephadex C-50, gel filtration on Sephadex G-150 and anion-exchanger DEAE-Sephadex A-50 (Figs. 1, 2, 3) in that order. The use of cation exchange chromatography (Fig. 1) at pH 5.87 separated DPP III, aminopeptidase M and some other unknown proteins/proteases from DPP IV as these are eluted in unadsorbed fractions. The proteinases DPP IV and DPP II were eluted in almost the same fraction at 0.75 *M* NaCl, as revealed by hydrolyses of Gly-Pro-4mβNA and Lys-Ala-4mβNA, respectively. These were subsequently separated on DEAE-Sephadex A-50 (Fig. 3) at pH 8.0. In this process, a pure form of DPP IV was obtained.

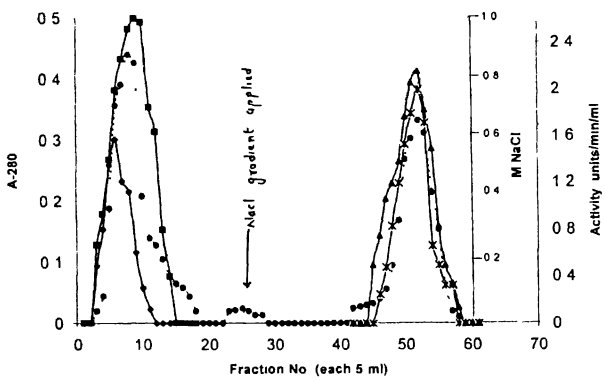


Fig. 1. Cation-exchange chromatography on CM-Sephadex C-50 [Ammonium sulfate fraction (20–50%, 20 ml) of goat brain homogenate was loaded on CM-Sephadex C-50 column at pH 5.87. Protein (•---•); Ala-βNA (■—■); Arg-Arg-4mβNA (◆—◆); Lys-Ala-4mβNA (*—*) and Gly-Pro-4mβNA (▲—▲), Ala-βNA for aminopeptidase M, Arg-Arg-4mβNA for DPP III, Lys-Ala-4mβNA for DPP II, Gly-Pro-4mβNA for DPP IV. The bound protein was eluted with a linear NaCl gradient (0–1.0 *M*)]

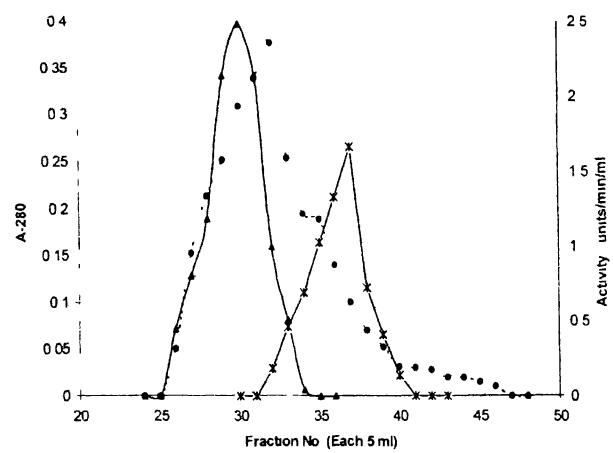


Fig. 2. Molecular sieve chromatography on Sephadex G-150 column [CM-Sephadex pool of DPP IV enzyme was loaded on Sephadex G-150 column equilibrated and eluted with 10 m*M* tris-HCl buffer pH 7.5 containing 1 m*M* EDTA and 0.2 *M* NaCl Protein (•---•); Lys-Ala-4mβNA (*—*) and Gly-Pro-4mβNA (▲—▲)]

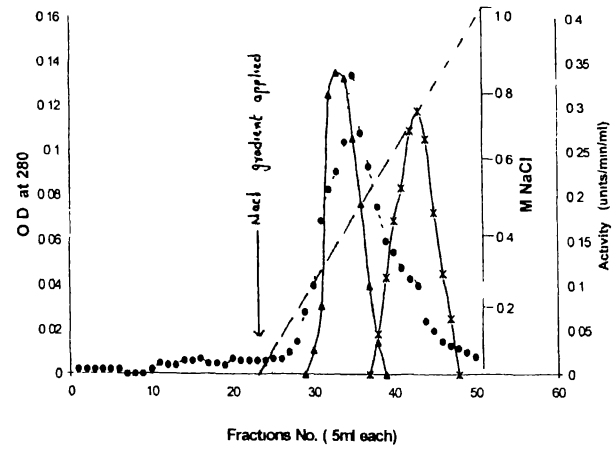


Fig. 3. Anion-exchange chromatography on DEAE-Sephadex A-50 [Sephadex G-150 pool of DPP IV enzyme was loaded on DEAE-Sephadex column equilibrated with 10 m*M* tris-HCl buffer pH 8.0. The bound protein was eluted with a linear NaCl gradient (0–1 0 *M*). Protein (•---•); Lys-Ala-4mβNA (*—*) and Gly-Pro-4mβNA (▲—▲)].

Table 1 shows a data of purification procedure of goat brain DPP IV which is purified approximately ~133-fold with a ~1.12% yield having specific activity of 29.2 units/mg with synthetic chromogenic substrate Gly-Pro-4mβNA. DPP IV proteinase isolated by this procedure was found to be electrophoretically homogeneous as evidenced by a single band at pH 8.4 (Fig. 4). This enzyme gave a single band on SDS-PAGE in the presence of β-ME (Fig. 5). The overall yield of DPP IV was approximately 440 μg from 200 g of goat brain. The mol. wt. of this enzyme was estimated to be ~65000 daltons in the presence of β-ME. However, its mol. wt. was ~200000 daltons in non-denaturing conditions i.e. on PAGE and Sephadex G-200 molecular sieve chromatography. The native enzyme is thought to be composed of 3 identical subunits. Earlier workers have reported the molecular wight value of 200000 daltons for this enzyme[24,25]. However, these workers did not show any band of ~65000 daltons on SDS-PAGE in their preparations.

Table 1. Purification of DPP IV proteinase

[Gly-Pro-4mβNA was used as substrate to measure the activity of DPP IV enzyme as described under 'Experimental'. The activity units are expressed in nmoles of 4-methoxy-β-naphthylamine liberated per min at 37°]

Purification steps	Total activity units (nmoles/min)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract supernatant, S_1	1158	5263	0.220	100	1
Acid fractionation supernatant, S_2	1056	2487	0.425	91.2	1.93
20–50% $(NH_4)_2SO_4$ pellet	246	86.2	2.853	21.24	12.96
CM-Sephadex C-50 pool pH = 5.87	90	8.82	10.201	7.77	46.36
Sephadex G-150 pool	40	2.6	15.001	3.45	68.22
DEAE-Sephadex A-50 pool pH = 8.0	13	0.44	29.240	1.12	132.91

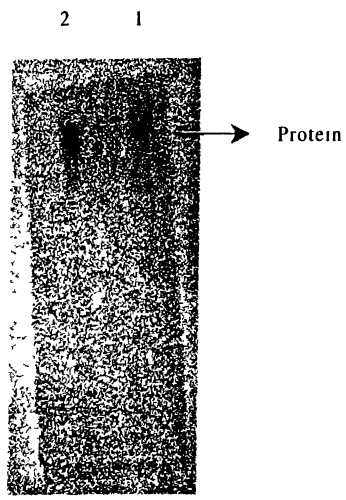


Fig. 4. Davis gel electrophoresis at pH 8.4 [30 μg and 40 μg of purified DPP IV enzyme was loaded in lanes 1 and 2, respectively and stained with Coomassie brilliant blue R-250].

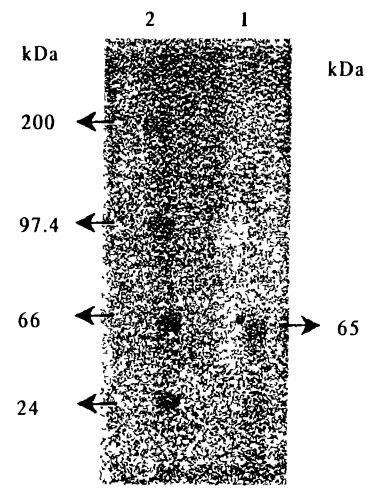


Fig. 5. Determination of molecular mass of goat brain DPP IV enzyme by SDS-PAGE at pH 7.2 : [Lane 1, DPP IV enzyme (40 μg), 65 kDa; lane 2, protein markers, (20 μg each of β-amylase, 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; and trypsinogen, 24 kDa)].

Substrate specificity :

Among the various dipeptidyl substrates tested at pH 8.0, DPP IV exhibited high activity towards the synthetic substrate Ala-Pro-4mβNA followed by the Phe-Pro-4mβNA and Gly-Pro-4mβNA in that order. The DPP II substrate Lys-Ala-4mβNA was cleaved to a negligible extent (Table 2). Furthermore, the purified enzyme was revealed not to be an aminopeptidase, because none of the aminopeptidase substrates Ala-βNA, Met-βNA, Val-βNA, Pro-βNA were hydrolyzed and no endopeptidase substrates such as Z-Phe-Arg-4mβNA, Bz-D,L-Arg-βNA were hydrolyzed. Thus, the enzyme preferentially cleaves a dipeptide X-Proline from the N-termini of peptides. In the present study, Gly-Pro-4mβNA has been established as a good chromogenic synthetic substrate for DPP IV proteinase. The Michaelis constant K_m and V_{max} values for hydrolysis of Gly-Pro-4mβNA by the goat brain DPP IV enzyme were obtained as 0.5 m*M* and 0.74 units/mg protein respectively. This K_m value was almost similar to the value reported for the enzyme from

Table 2. Substrate specificity of DPP IV proteinase with -4mβNA and -βNA substrates

[The final concentration of each substrate in the assay mixture was 0.2 m*M*. The activity of DPP IV proteinase with Gly-Pro-4mβNA was taken as 100% and the percent activities with other substrates were calculated relative to this substrate. The results are an average of three different experiments conducted at different times]

Substrate	Activity units (nmoles/min/ml)	Activity (%)
H-Gly-Pro-4mβNA	1.756	100
H-Ala-Pro-4mβNA	2.220	126.4
H-Phe-Pro-βNA	1.883	107.5
H-Lys-Ala-4mβNA	0.136	7.74

human kidney soluble fraction[26].

Effect of inhibitors :

Various standard proteinase inhibitors against DPP IV were examined (Table 3). DPP IV was strongly inhibited by PMSF and moderately by α_1-antitrypsin and soybean trypsin inhibitor type II. However, it was not inhibited by such proteinase inhibitors as leupeptin, pepstatin (inhibitor of cathepsins) and bestatin (as inhibitor of aminopeptidase), EDTA, 1,10-phenanthroline (inhibitor of metalloproteinase). The enzyme activity was strongly inhibited competitively by Ala-Pro and His-Pro as substrate analogs. Sulfhydryl reagents like β-ME, iodoacetic acid, iodoacetamide did not affect the enzyme activity. These findings indicated that a SH-residue is not involved in the expression or regulation of the activity of DPP IV. Among the metal ions, Zn^{2+} was found to be the strongest inhibitor whereas Ni^{2+} and Ca^{2+} inhibited the enzyme moderately. These findings also indicate that metal-binding region of DPP IV molecule is important for regulation of the enzyme activity. However, the localization of metal binding region on the DPP IV molecule has not been identified. These results of inhibition studies indicated that the active-centre of the purified DPP IV proteinase contains serine residues.

Table 3. Effect of various inhibitors on DPP IV proteinase

[Activity of DPP IV proteinase was measured with Gly-Pro-4mβNA at pH 8.0. Enzyme was first activated by incubating for 5 min at 37° and then this activated enzyme was pre-incubated with the respective inhibitor for 10 min and then the residual enzyme activity was determined by the standard assay procedure for DPP IV. The inhibition was taken as 0% when no inhibitor was added]

Inhibitor	Final concentration (m*M*)	Inhibition %
None	–	0.0
Iodoacetic acid	1.0	19.5
	0.1	0
Benzamidine.HCl	1.0	7.1
	0.1	0
TPCK	1.0	84.8
	0.1	79.9
p-Hydroxy mercuriphenylsulfonic acid	1.0	39.6
N-Ethylmaleimide	1.0	4.8
	0.1	0
TLCK	1.0	89.9
	0.1	69.2
Iodoacetamide	1.0	10.8
	0.1	0
Puromycin	1.0	88.5
	0.1	80.9
Aprotinin	1.0	90.1
	0.1	85
2,4,6-Trinitrobenzenesulfonic acid trihydrate	1.0	75.9
	0.1	39.8
p-Aminophenylmercuric acetate	1.0	28.9
	0.1	0
Soybean trypsin inhibitor type II	1.0	56.8
	0.1	30
α_1-Antitrypsin	1.0	57.9
	0.1	22.5
PMSF	1.0	100
EDTA	1.0	0
Antipain	1.0	0
Bestatin	1.0	0
Pepstatin	1.0	0
1,10-Phenanthroline	1.0	0
Leupeptin	1.0	8.9
Zinc acetate	1.0	100
Zinc actetate + EDTA (1 m*M*)	1.0	0
$ZnCl_2$	1.0	91.8
$ZnCl_2$ + EDTA (1 m*M*)	1.0	0
$FeSO_4$	1.0	0
$MgSO_4$	1.0	20.9
$MgSO_4$ + EDTA (1 m*M*)	1.0	0
SDS	1%	100
Triton X-100	1%	58.4

pH optimum and stability :

The maximum hydrolytic activity of goat brain DPP IV proteinase with Gly-Pro-4mβNA as substrate was at pH 8.0. The enzyme was found to be stable between pH 6.8 to 9.0 and was unstable below pH 5.5 and above pH 10.0.

Temperature stability and optimum :

The purified enzyme was incubated at various temperatures (0–80°) for 10 min and the residual activity was measured by the assay procedure described in 'Experimental'. This enzyme was stable fairly well upto 50° and thereafter the activity decreases suddenly due to thermal denaturation of enzyme protein. At 55°, the enzyme retained ~58% activity and at 60°, ~34% activity could be retained. There was complete inactivation at 70°. This data indicates that the goat brain enzyme is heat labile. In contrast, the enzyme from lamb kidney[27], didn't lose its activity by heating at 60° even for 15 min.

For determining the temperature optima, the enzymic activity was measured at different temperatures (0–80°) by the assay procedure given in 'Experimental'. The optimal tem-

perature for hydrolytic activity of DPP IV enzyme with Gly-Pro-4mβNA as substrate was ~50°. This proteinase could exhibit only ~13% activity at 20°. There was a gradual increase upto 50° and thereafter the denaturation of enzyme occurred very rapidly. The activation energy, E_a for the hydrolysis of Gly-Pro-4mβNA was determined using Arrhenius plot and it was found to be ~11 kcal/mol.

Effect of urea :

This enzyme was highly stable in presence of urea retaining full activity even when the enzyme was incubated with 8*M* urea for 1 h.

Storage stability :

The DPP IV proteinase stored at 4° in its active form in 25 m*M* tris-HCl buffer (pH 7.5) having 1 m*M* EDTA retained 100% activity upto at least 3 weeks. However, it lost 50% activity in the 4th week and 100% activity in the 6th week of storage time.

Conclusion :

In the present study, a high mol. wt. DPP IV enzyme has been isolated, purified and characterized from goat brain tissue. This is a serine proteinase as indicated by its inhibition studies. Goat brain DPP IV requires neither metals nor any other cofactors for its activity.

Experimental

All the chemicals used were of highest purity available. The -β-naphthylamide, -4-methoxy-β-naphthylamide substrates like Gly-Arg-4mβNA, Gly-Pro-4mβNA, Gly-Ala-βNA, Ala-Pro-4mβNA, Phe-Pro-βNA, Lys-Ala-4mβNA, Z-Phe-Arg-4mβNA, Bz-D,L-Arg-βNA, Bz-Phe-Val-Arg-4mβNA, Pro-βNA, Gly-βNA, β-naphthylamine, and 4-methoxy-β-naphthylamine were purchased from Bachem Feinchemikalein AG, Bubendorf, Switzerland.

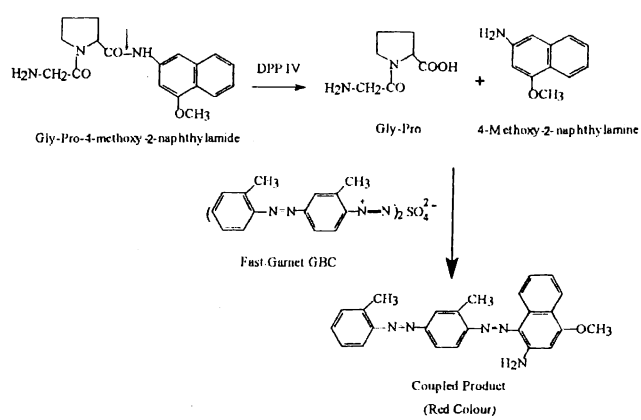
Bovine serum albumin (BSA), phosphorylase *b*, α_1-antitrypsin, PMSF, soybean trypsin inhibitor type-II, Fast Garnet GBC, *p*-hydroxymercuribenzene sulphonic acid, *N*-hydroxysuccinimide, iodoacetamide, benzimidine.HCl, puromycin, pepstatin, leupeptin, antipain, bestatin, α-*N*-tosyllysine chloromethyl ketone (TLCK), α-*N*-tosylphenylalanine chloromethyl ketone (TPCK), Coomassie brilliant blue G and Blue Dextran-2000® were supplied by Sigma Chemical Co., St. Louis, MO USA. *N,N*-Methylene-bis-acrylamide and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from FERAK Berlin, West Germany. Acrylamide (electrophoresis grade) was procured from John Baker Inc., Colorado, USA and ammonium persulphate from May and Baker Ltd., Dageham, England. Glycine and tris-HCl were purchased from Glaxo Laboratories, India. β-Mercaptoethanol was supplied by Loba Chemical Company, India. Ammonium sulphate and urea were purchased from S D Fine-Chem. Ltd., Mumbai. Sucrose and sodium dodecyl sulfate (SDS) were procured from Himedia Laboratories, Mumbai. Sephadex G-150, CM-Sephadex C-50 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Sweden.

Refrigerated centrifuge IEC-25 and table top centrifuge Remi R8C were used for routine centrifugation. Systronics Spectrophotometer 108 was used for recording the absorbance in UV/VIS range.

Protein samples were concentrated using Ultrafiltration Amicon Cell Model 8200 having YM10 membrane under compressed nitrogen pressure of 4–5 psi.

Assay for dipeptidylpeptidase IV :

A simple colourimetric method for estimating DPP IV activity in all purification steps was used. The enzyme 200 μl was added to the assay buffer 750 μl; 50 m*M* tris-HCl, pH 8.0 and pre-incubated for 5 min at 37°. The enzymatic reaction was started by adding the substrate stock solution (50 μl, Gly-Pro-4mβNA, 4 mg/ml DMSO). After 60 min incubation, the reaction was stopped by adding 500 μl stopping reagent (1.0 *M* sodium acetate buffer, pH 4.0) and then 500 μl of Fast Garnet GBC (1.0 mg/ml in H_2O) was added. After 10 min, the red coloured dye was extracted with n-butanol (2.0 ml) and estimated at 520 nm. Both enzyme and substrate blanks were also included. In case of enzyme blank, substrate was added after terminating the reaction, similarly in substrate blank, enzyme was added after termination of the reaction. One unit of enzyme activity is defined as the amount of enzyme which released 1 nmole of 4-methoxy-β-naphthylamine from Gly-Pro-4mβNA substrate at 37° per min under the assay conditions.



DPP IV catalyzed reaction – an assay procedure chemistry.

Assays for enzymes aminopeptidase M [EC 3.4.11.2], DPP II [EC 3.4.14.2] and DPP III [EC 3.4.14.4] were carried out using Ala-βNA[28], Lys-Ala-4mβNA[29] and Arg-Arg-4mβNA[28], respectively as the substrates.

Protein estimation :

Protein in crude and purified samples was estimated by Lowry *et al.*[30] method using bovine serum albumin as the standard protein.

Purification :

All the procedures were carried out at 4° unless stated otherwise. The fresh goat brain (200 g) was homogenized in 1000 ml of cold 0.1 *M* tris-HCl buffer (pH 7.5) containing 0.2 *M* NaCl and 1 m*M* EDTA (buffer A), stirred for 1 h and centrifuged at 12000 × g for 20 min. The supernatant S_1, was acidified by drop-wise addition of 1 *N* HCl to pH 6.0 and allowed to autolyze at 25° for 6 h and centrifuged again at 12000 × g for 20 min. The supernatant S_2, was saturated upto 20% with solid $(NH_4)_2SO_4$ while stirring and then centrifuged at 12000 × g for 20 min. The supernatant S_3, was saturated upto 50% with solid $(NH_4)_2SO_4$ and again centrifuged at 12000 × g for 20 min.

The pellet obtained above was dissolved in minimum volume of 50 m*M* sodium acetate buffer, pH 5.87 (buffer B), dialyzed extensively and then concentrated through Amicon Diaflo Ultra-filtration cell using YM-10 membrane. This dialyzed enzyme concentrate was loaded on cation-exchanger CM-Sephadex C-50 equilibrated with buffer B. After washing the column free from unadsorbed proteins, the bound proteins were eluted by applying a linear NaCl gradient (0.0–1.0 *M* NaCl). In this step, DPP IV proteinase was eluted at 0.75 *M* NaCl concentration. The fractions showing DPP IV proteinase activity were pooled, concentrated and dialysed against 10 m*M* tris-HCl buffer (pH 7.5) containing 1 m*M* EDTA and 0.2 *M* NaCl (buffer C).

The concentrated pool of activity obtained above was fractionated on Sephadex G-150 column with eluent buffer C. The fractions eluted from the column were assayed for DPP II and DPP IV proteinases. The fractions having DPP IV proteinase activity were again concentrated and dialysed against 10 m*M* tris-HCl buffer, pH 8.0 (buffer D) and the pool was loaded on the anion exchanger DEAE-Sephadex A-50 column equilibrated with buffer D. After washing the column free from unadsorbed proteins, the DPP IV was eluted by applying a linear NaCl gradient (0.0–1.0 *M* NaCl). The fractions having DPP IV proteinase activity eluted at 0.3 *M* NaCl concentration, were pooled and concentrated. The concentrated DPP IV enzyme was stored in 25 m*M* tris-HCl buffer pH 7.5 containing 1 m*M* EDTA and 10% glycerol at 4°. The purity of the enzyme was established by gel electrophoresis at pH 8.4[31].

The molecular mass of the enzyme was determined by gel filtration on analytical Sephadex G-200 column and by SDS-PAGE at pH 7.2[32].

Acknowledgement

Financial assistance received from Kurukshetra University, Kurukshetra in the form of University Research Scholarships to Ashwani Mittal and Shiwani Khurana during the course of this work is gratefully acknowledged.

References

1. V. K. Hopsu-Havu and G. G. Glenner, *Histochemie*, 1966, **7**, 197.
2. J. Heins, P. Welker, C. Schonlein, T. Born, B. Hartrodt, K. Neubert, D. Tsuru and A. Barth, *Biochim. Biophys. Acta*, 1988, **954**, 161.
3. R. Mentlein, *Regulatory Peptides*, 1999, **85**, 9.
4. I. Nausch, R. Mentlein and E. Heymann, *Biol. Chem. Hoppe-Seyler*, 1990, **371**, 1113.
5. L. A. Frohmann, T. R. Down, E. P. Heimer and A. M. Felix, *J. Clin. Invest.*, 1989, **83**, 1533.
6. M. D. Gorrell, V. Gysbers and G. W. Mccaughan, *Scand. J. Immunol.*, 2001, **54**, 249.
7. M. Hagihara, M. Ohhashi and T. Nagatsu, *Clin. Chem.*, 1987, **33**, 1463.
8. S. Tanaka, T. Murakami, H. Horikawa, M. Sugiura, K. Kawashima and T. Sugita, *Int. J. Immunopharmacol.*, 1997, **19**, 15.
9. M. Maes, I. De. Meester, S. Scharpe, R. Desnyder, R. Ranjan and H. Y. Meltzer, *Acta Psychiatr. Scand.*, 1996, **93**, 1.
10. J. J. Holst and C. F. Deacon, *Diabetes*, 1998, **47**, 1663.
11. C. Callebaut, B. Krust, E. Jacotot and A. G. Hovanessian, *Science*, 1993, **262**, 2045.
12. A. J. Kenny, A. G. Booth, S. G. George, J. Ingram, D. Kershaw, E. J. Wood and A. R. Young, *Biochem. J.*, 1976, **157**, 169.
13. B. Schmauser, G. Kilian, W. Reutter and R. Tauber, *Glycobiology*, 1999, **9(12)**, 1295.
14. S. Hartel, C. Hanski, W. Kreisel, C. Hoffmann, J. Mauck and W. Reutter, *Biochim. Biophy. Acta*, 1987, **924**, 543.
15. Y. Ikehara, S. Ogata and Y. Misumi, *Methods Enzymol.*, 1994, **244**, 215.
16. K. Kojima, T. Hama, T. Kato and T. Nagatsu, *J. Chromatog.*, 1980, **189**, 233.
17. I. Ohkubo, K. Huang, Y. Ochiai, M. Takagaki and K. Kani, *J. Biochem.*, 1994, **116**, 1182.
18. S. I. Egawa, Y. Watanabe, Y. Kikuya and Y. Fujimoto, *J. Biochem.*, 1998, **124**, 428.
19. H. G. Bernstein, E. Schön, S. Ansorge, I. Röse and A. Dorn, *Int. J. Devel. Neurosci.*, 1987, **5**, 237.

20. G. Kreil, L. Haiml and G. Suchanek, *Eur. J. Biochem.*, 1980, **111**, 49.
21. C. Bordallo, J. Schwencke and M. S. Rendueles, *FEBS Lett.*, 1984, **173**, 199.
22. A. Banbula, M. Bugno, J. Goldstein, J. Yen, D. Nelson, J. Travis and J. Potempa, *Inf. and Immun.*, 2000, **68(3)**, 1176.
23. A. Davy, K. K. Thomsen, M. A. Juliano, L. C. Alves, L. Svendsen and D. J. Simpson, *Plant Physio.*, 2000, **122**, 425.
24. Puschel, R. Mentlein and E. Heymann, *J. Biochem.*, 1982, **126**, 359.
25. G. B. Wolf, J. E. Scherberich, P. Fischer and W. Scheppe, *Clinica Chimica Acta*, 1989, **179**, 61.
26. D. Mantle, *Clinica Chimica Acta*, 1991, **196**, 135.
27. T. Yoshimoto and R. Walter, *Biochim. Biophys. Acta*, 1977, **485**, 391.
28. T. V. Perttula, *Clinica Chimica Acta*, 1988, **177**, 179.
29. P. Garg, N. Raghav, S. Pal, R. C. Kamboj and H. Singh, *J. Indian Chem. Soc.*, 1991, **68**, 589.
30. O. H. Lowry, N. J. Rosebrough, N. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
31. B. J. Davis, *Annals N. Y. Acad. Sci.*, 1964, **121**, 404.
32. K. Weber and M. Osborn, *J. Biol. Chem.*, 1969, **244**, 4406.