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Purification and characterization of a dipeptidyl peptidase 9-like enzyme from bovine testes

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1. ABSTRACT

Until now, only recombinant forms of dipeptidyl peptidase (DPP) 8 and 9 have been characterized. We purified non DPPII-non DPPIV enzymes from a natural source. A first DPP8/9-like enzyme was enriched 1160-fold from bovine testes and identified as 'DPP9-like enzyme' by using an anti-DPP9 antibody. A second 576-fold enriched preparation ('DPP enriched peak 3') also showed DPP8/9-like activity. SDS-PAGE analysis showed that the DPP9-like enzyme had a monomeric molecular mass of approx. 100 kDa. Size exclusion chromatography generated a native molecular mass of 164 kDa for the DPP9-like enzyme and one of 234 kDa for the DPP enriched peak 3, suggesting that both proteins appeared to be dimeric. Both enriched preparations and rDPP8 showed roughly similar substrate specificity and inhibitor profiles. The DPP9-like enzyme and the DPP enriched peak 3 possessed a neutral pH optimum and were stable at -80°C. We can conclude that the natural DPP9-like enzyme and the DPP enriched peak 3 are closely related to the recombinant forms of human DPP9 and DPP8.

2. INTRODUCTION

Dipeptidyl peptidases (DPPs), such as DPPIV, fibroblast activation protein alpha (FAP), DPPII, DPP8 and DPP9, cleave N-terminal dipeptides from peptides with proline at the penultimate position (1). These enzymes are all members of the same clan SC of serine proteases as based on similarities in three-dimensional structure, arrangement of the catalytic residues (Ser, Asp, His) and the primary sequence around their catalytic residues (Gly-X-Ser-X-Gly where X is any amino acid) (2). In 2000, human DPP8 and DPP9 were identified *in-silico* by Abbott and coworkers (3). DPP8 is localized on chromosome 15q22 and DPP9 on 19p13.3. While both genes are encoded on different chromosomes, their amino acid sequences are very homologous (79% homology) (4). DPP8 and DPP9 share a 58% overall identity and a 72% identity in the 200 amino acid fragment containing the presumed catalytic region. This high level of identity suggested that they may have arisen by gene duplication (5).

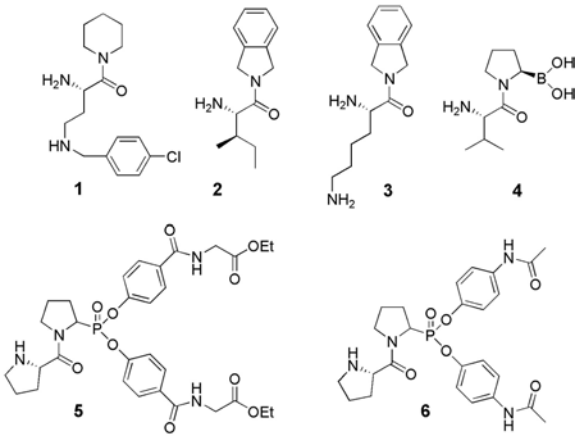

Figure 1. Structures of the DPP inhibitors used. The DPPII inhibitor UAMC00039 (1), the DPP8/9 inhibitors *Allo*-Ile-isoindoline (2) and Lys-isoindoline (UAMC00071) (3), the DPP inhibitor Val-boroPro (4), the DPPIV/8/9 inhibitor AB207 (5) and the DPPIV inhibitor AB192 (6).

Different splice variants of DPP8 and DPP9 have been identified; their biological significance is unknown (3, 4, 6). Activity was observed only for the full-length variants of the two recombinantly expressed proteins of 882 and 892 amino acids for DPP8 and DPP9, respectively. Both enzymes are catalytically active as homodimers and have a molecular mass between 95 and 110 kDa on SDS-PAGE (7, 8). There is discussion about the enzymatic activity of the 863 amino acid variant of DPP9. Ajami *et al.* and Qi *et al.* reported this short rDPP9 form as enzymatically active in contrast to Olsen *et al.* and Bjelke *et al.* who only found enzymatic activity for the 892 amino acid DPP9 (4, 5, 7, 9). Lee *et al.* reported that the C-terminal loop is important for the enzymatic activity and/or the quaternary structure of DPP8. This may explain why addition of either V5 or His tag to the C-terminal region of DPP8 or DPP9 produces only a monomeric form or inactivates the enzyme in another way (3, 4, 7, 8). In fact, the C-terminal loop is also important for the enzymatic activity of DPPIV (10, 11).

Like DPPIV, DPP8 and DPP9 have an ubiquitous mRNA expression pattern. The highest DPP8 mRNA levels are found in testis and placenta. It is upregulated in activated T cells and expressed in all B and T cell lines examined (3). The DPP9 mRNA expression levels are highest in skeletal muscle, heart, liver and peripheral blood leukocytes, and lowest in brain (4, 5). DPP8 and DPP9 differ from DPPIV and FAP in that they contain no transmembrane domain. DPP8 is not N- or O-linked glycosylated. DPP9, in contrast, has two potential N-linked glycosylation sites (3-5, 9).

Recombinant DPP8 and DPP9 show very similar Michaelis-Menten kinetics and substrate specificity. They hydrolyze the chromogenic DPPIV substrates Gly-Pro-, Ala-Pro- and Arg-Pro-pNA. The catalytic efficiencies towards these substrates are generally lower than those of DPPIV. Both enzymes have the ability to cleave glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), neuropeptide Y (NPY) and peptide YY (PYY) *in vitro*. Because of the proposed intracellular cytoplasmic localization of the peptidases, it remains questionable whether the *in vitro* observed proteolytic cleavage of these hormones by DPP8 and DPP9 is of relevance with respect to *in vivo* physiological functions (7).

No clear physiological role of DPP8 and DPP9 has been determined. It is even possible that these DPPs are responsible for some of the many functions attributed to DPPIV. A selective DPP8/9 inhibitor, reported by Lankas *et al.*, attenuated proliferation and IL-2 release in *in vitro* models of immune responses in human T cells upon stimulation, functions that were previously assigned to DPPIV (12). Moreover, this inhibitor produced severe toxicity and various pathological symptoms in animals. Therefore, the selectivity of DPP inhibitors may also be important to ensure an optimal safety profile of potential clinical candidates (13).

So far, only recombinant forms of DPP8 and DPP9 have been characterized. In literature some controversy exists concerning the biochemical and enzymatic characteristics of these enzymes. DPP8 and DPP9 have not been purified or studied from any natural source. Therefore, the aim of this study is to investigate the natural form of these enzymes. Here, we report the purification, biochemical and enzymatic characterization of DPPIV-like enzymes from bovine testes.

3. MATERIALS AND METHODS

3.1. Materials

The DPPII inhibitor N-(4-chlorobenzyl)-4-oxo-4-(1-piperidinyl)-1,3-(*S*)-butanediamine dihydrochloride (UAMC00039), the DPP8/9 inhibitors (2*S*,3*R*)-2-amino-1-(isoindolin-2-yl)-3-methylpentan-1-one (*Allo*-Ile-isoindoline) and (*S*)-2,6-diamino-1-(isoindolin-2-yl)hexan-1-one (Lys-isoindoline; UAMC00071), the DPP inhibitor (*R*)-1-((*S*)-2-amino-3-methylbutanoyl)pyrrolidin-2-ylboronic acid (Val-boroPro), the DPPIV/8/9 inhibitor Bis{4-((ethoxycarbonyl)methylaminocarbonyl)phenyl} 1-((*S*)-prolyl)pyrrolidine-2(*R*,*S*)-phosphonate (AB207) and the DPPIV inhibitor Bis(4-acetamidophenyl) 1-((*S*)-prolyl)pyrrolidine-2(*R*,*S*)-phosphonate hydrochloride (AB192) were synthesized as described (12, 14-16) (Figure 1). Bovine testes were kindly provided by a local slaughterhouse. Fluorophosphate (FP)-biotin was synthesized by the department of Chemistry of the K.U. Leuven as described (17). Microcon centrifugal concentrators (YM-50) were from Millipore. DPPII and DPPIV were purified from human seminal plasma (18, 19). Recombinant human DPP8 (rDPP8) was expressed and purified as described (20). The anti-DPP9 antibody was generated by Eurogentec. The columns, chromatography media (DEAE Sepharose Fast Flow, concanavalin A (Con A)-Sepharose, HiTrap nickel-chelating HP (5 ml), HiTrap Phenyl HP (5 ml), Phenyl Sepharose CL-4B, HiTrap Q HP (1 ml and 5 ml), Superdex 200 HR 10/30 and HiTrap Protein A HP (1 ml)) and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased

from GE Healthcare. The mini-PROTEAN® 3 system, the mini trans-blot® cell assembly, the Molecular Weight Standards and nitrocellulose membranes were obtained from Bio-Rad. Fast Blue B salt was purchased from Serva. Lys-Ala-p-nitroanilide (-pNA), Ala-Pro-pNA, Gly-Pro-pNA, Arg-Pro-pNA and Gly-Pro-4-methoxy-2-naphtylamide (-4Me2NA) were obtained from Bachem. All other reagents were from Sigma.

3.2. Enzyme assays

Enzymatic activities were determined kinetically in a final volume of 200 µl for 10 min at 37°C by measuring the initial velocities of pNA release (405 nm) from the substrate using a Spectramax plus microtiterplate reader (Molecular Devices). One unit of enzymatic activity is the amount of enzyme that catalyzes the release of 1 µmol pNA from the substrate per min under assay conditions. DPPII activity was determined using 1 mM Lys-Ala-pNA in 0.05 M cacodylic acid-NaOH buffer, pH 5.5, containing 10 mM EDTA and 14 µg/ml aprotinin (18). 2 mM Ala-Pro-pNA in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 and 0.5 mM Gly-Pro-pNA or Arg-Pro-pNA in 0.05 M Tris buffer, pH 8.3, containing 0.1% Tween 20 were used to probe DPPIV, DPP8 and DPP9 activity. Protein concentration was determined according to Bradford (21) with BSA as the standard.

3.3. Preparation of bovine testis homogenate

All homogenization steps were performed at 4°C. Bovine testes were cut to pieces and mixed with a blender. The frozen (-80°C) mixture (175-220 g) was homogenized in 0.02 M Tris buffer, pH 7.4, containing 10 mM EDTA and 14 µg/ml aprotinin (5 ml/g). The homogenized tissue was centrifuged for 10 min at 3000 g and the resulting supernatant was subsequently centrifuged once during 30 min at 20000 g and finally 1 hour at the same speed. The final supernatant was filtered and stored at -80°C.

3.4. Purification of DPPs

Bovine testis homogenates (4.7 l) were quickly thawed at 37°C and loaded onto a DEAE Sepharose Fast Flow column (412 ml), equilibrated and washed with 0.02 M Tris buffer, pH 7.4. Elution was performed with a linear 0-0.5 M NaCl gradient in equilibration buffer. Fractions of peak 3 that contained DPP activity were stored at -80°C. The active fractions of peak 1 were pooled and applied to a Con A-Sepharose column (42 ml), equilibrated and washed with 0.02 M Tris buffer, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, 14 µg/ml aprotinin and 0.5 M NaCl (18). Elution was performed with 1 M methyl-alpha-D-glucopyranoside in the same buffer. Flow through fractions containing DPP activity were brought to 5 mM imidazole and loaded onto a HiTrap nickel-chelating HP column (5 ml), equilibrated and washed with 0.02 M Tris buffer, pH 7.4, containing 5 mM imidazole and 0.5 M NaCl. Elution was performed with a linear 0.005-0.5 M imidazole gradient in the same buffer. To fractions containing DPP activity 0.5 M $(NH_4)_2SO_4$ was added. The sample was incubated for 1 hour on ice and subsequently centrifuged for 90 min at 9000 g at 4°C. The supernatant was loaded onto a 5 ml HiTrap Phenyl HP column, equilibrated and washed with 0.02 M Tris buffer, pH 7.4, containing 0.5 M $(NH_4)_2SO_4$. Elution was performed with a linear 0.5-0 M $(NH_4)_2SO_4$ gradient. Active fractions were diluted in 0.02 M Tris buffer, pH 7.4, and subsequently loaded on a HiTrap Q HP column (1 ml), equilibrated with the same buffer. Elution was performed with a linear 0-1 M NaCl gradient in equilibration buffer. Fractions containing DPP activity were applied to 200 µl Lys-isoindoline affinity gel, prepared as described in (22) and washed (3x) with 0.02 M Tris buffer, pH 7.4, containing 0.150 M NaCl. Sample (1 ml at a time) was incubated with the affinity gel for 1 hour at room temperature. After all the sample was loaded, the gel was washed (4x) with equilibration buffer. Elution was carried out by applying 200 µl of 2 mM Lys-isoindoline (UAMC00071) in 0.02 M Tris buffer, pH 7.4, to the gel for 1 hour at room temperature. The inhibitor was removed adequately by subsequent cycles of concentration and dilution in 0.02 M Tris buffer, pH 7.4, using Microcon centrifugal concentrators with a nominal molecular weight cut-off of 50 kDa. Finally, the Lys-isoindoline gel was treated with SDS-PAGE loading buffer and boiled for 5 min.

The active fractions of peak 3 of the DEAE Sepharose Fast Flow were quickly thawed at 37°C and subjected to the same purification procedure as peak 1. This included application to a lectin-affinity, a nickel-chelating, a Phenyl Sepharose and HiTrap Q HP column under identical conditions as used for the purification of peak 1. After the anion-exchange chromatography, fractions containing DPP activity were concentrated using a Microcon centrifugal concentrator YM-50. From now on we will call this preparation 'DPP enriched peak 3'. The sample was subjected to the Lys-isoindoline affinity chromatography, based on the purification of peak 1 with slight modifications. The gel was washed (3x) with 0.02 M Tris buffer, pH 7.4, containing 1 M NaCl and elution was performed by loading 2 mM Lys-isoindoline (UAMC00071) in the same buffer on the gel.

3.5. Estimation of molecular mass by SDS gelelectrophoresis and size exclusion chromatography

SDS-PAGE (7.5%) was carried out in the presence of 2-mercaptoethanol essentially according to the Laemmli method in 0.025 M Tris, 0.192 M glycine, pH 8.3, containing 0.5% (w/v) SDS (23). Broad-range molecular weight marker proteins (myosin (195/212 kDa), beta-galactosidase (117/121 kDa), BSA (97/100 kDa) and ovalbumin (50/54 kDa)) were used. Gels were stained with Coomassie blue.

Active fractions obtained from HiTrap Q HP chromatographies were injected onto a Superdex 200 HR 10/30 size exclusion column, equilibrated with 0.02 M Tris buffer, pH 7.4, containing 0.15 M NaCl at a flow rate of 0.5 ml/min. For the native molecular mass determination of the samples, the partition coefficient K_{av} was plotted against log (molecular mass) and a calibration curve with thyroglobulin (molecular mass, 669 kDa), ferritin (molecular mass, 440 kDa), aldolase (molecular mass, 158 kDa), BSA (molecular mass, 67 kDa) and ovalbumin (molecular mass, 43 kDa) was used. Blue dextran (molecular mass, 2000 kDa) was an indicator for the void volume.

3.6. Anti-DPP9 antibody generation

A 15 amino acid containing, immunogenic, unique sequence found in DPP9 (LKSQGYDWSEPFSPG) was selected based on hydrophobicity (24) and the occurrence of beta-turns (25). The occurrence of the sequence in other mammalian proteins was checked using the Blast program. The sequence was synthesized with an amino-terminal cysteine residue and coupled to keyhole limpet hemocyanin (KLH) (Eurogentec). After immunisations (day 0, 21, 49), the rabbits were bled at day 59. Preimmune and immune sera were collected and the IgG fractions were purified using HiTrap Protein A HP affinity chromatography.

3.7. Western blot analysis

Samples were separated on SDS-PAGE (7.5%) as described above and the proteins were transferred by electroblotting onto a nitrocellulose membrane in 0.025 M Tris, 0.192 M glycine, pH 8.3, containing 20% methanol using the mini trans-blot cell assembly. After blocking with 0.05 M Tris buffer containing 0.15 M NaCl, 5 mM NaN_3 and 2% Tween 20 overnight at 4°C, blots were incubated with the anti-DPP9 antibody for 2 hours at room temperature. In the control experiment, preimmune purified IgGs were used. Goat anti-rabbit Ig conjugated with horseradish peroxidase (HRP) (Biosource) was used as secondary antibody and the incubation was carried out for 1 hour at room temperature. After each incubation step the blots were washed (4x) in 0.05 M Tris buffer containing 0.15 M NaCl and 0.1% Tween 20. Visualization of the blots occurred by the Lumi-imager (Boehringer Mannheim) using ECL Western blotting detection reagents.

3.8. Native gelelectrophoresis and enzymatic staining

For native gel electrophoresis we used the same procedure as described above but we omitted SDS from all the solutions and 2-mercaptoethanol from the loading buffer and did not boil the samples. Kaleidoscope prestained markers (myosin (199 kDa), beta-galactosidase (128 kDa) and BSA (85 kDa)) were used. After electrophoresis under non-denaturing conditions, DPP activity was detected. The gel was covered with a nitrocellulose membrane saturated with 5 mM Gly-Pro-4Me2NA in 0.05 M HEPES buffer, pH 7.4, containing 10% DMSO. Incubation was performed overnight at room temperature. Finally, enzymatic activity was visualized by a Fast Blue B salt solution (4 mg/ml in 0.05 M sodium phosphate, pH 7.5).

3.9. Effect of detergents and reducing agents

Samples were diluted in PBS (Gibco BRL, Life Technologies) with or without the addition of Tween 20 (0.1%), dithiothreitol (DTT) (1-10 mM) or 2-mercaptoethanol (0.05-0.125-0.2%). After 0 min, 2 hours and 1 day at 4°C, DPP activity towards Ala-Pro-pNA was assayed as described above. To test the influence of reducing agents, the Ala-Pro-pNA-cleaving activity was measured in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 with or without addition of 10 mM EDTA and 14 µg/ml aprotinin.

3.10. Kinetic properties

Samples were diluted in PBS containing 0.1% Tween 20. Activity towards dipeptide-derived chromogenic substrates was assayed as described above. The values for K_m and V_{max} were determined by plotting the initial velocities of product formation against different substrate concentrations. At least six different concentrations of substrate in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 were used. The data were fitted to the Michaelis-Menten equation by non-linear regression analysis using GraFit version 5 (26).

3.11. Inhibition measurements

Inhibition of DPP activity was analysed spectrophotometrically as described here, except that the DPP inhibitor was preincubated with the enzyme for 15 min at 37°C before adding the substrate. Other inhibitors tested included the serine protease inhibitors di-isopropyl fluorophosphate (DFP) (1 mM), aprotinin, and the metal chelating agent EDTA. 14 µg/ml aprotinin and/or 10 mM EDTA were added to 0.05 M HEPES buffer, pH 7.4, and 0.05 M Tris buffer, pH 8.3. Samples were diluted in PBS. Activity towards Ala-Pro-pNA and Gly-Pro-pNA were assayed as described above. IC_{50} values were obtained with substrate concentrations near the K_m value (hydrolysis of Ala-Pro-pNA (150 µM) in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 by rDPP8 diluted in PBS containing 0.1% Tween 20) and at least ten different inhibitor concentrations were used. IC_{50} values were calculated using GraFit version 5 (26).

3.12. Reaction with fluorophosphate (FP)-biotin and detection of FP-biotin reactivity

Reactions between protein samples and FP-biotin were performed based on (17). Samples were dissolved in 19.5 µl of 0.02 M Tris buffer, pH 7.4. FP-biotin (200 µM in DMSO) was added to a final concentration of 5 µM and the reactions were incubated at 37°C for 30 min. Reactions were quenched by adding reducing SDS-PAGE loading buffer and boiled for 5 min. These samples were run on SDS-PAGE (7.5%) and transferred by electroblotting onto a nitrocellulose membrane as described above. Blocking and washing solutions were as described above. Blots were incubated with HRP conjugated avidin (Dako) for 1 hour at room temperature and visualized by the Lumi-imager using ECL Western blotting detection reagents.

3.13. pH profile

DPP activity was measured over the pH range 4.0-9.2 (in increments of 0.25) using a buffer containing 0.025 M ethanoic (acetic) acid, 0.025 M cacodylic acid, 0.025 M HEPES, 0.025 M Tris and 0.1 M NaCl, titrated to the desired pH with NaOH or HCl. The influence of pH on the hydrolysis rate was studied using the substrate Ala-Pro-pNA (1 mM). Active fractions obtained from HiTrap Q HP chromatographies were diluted in 0.02 M Tris buffer, pH 7.4, containing 0.1% Tween 20 and 1 mM DTT.

4. RESULTS

4.1. Purification of non DPPII-non DPPIV enzymatic activity and identification as DPP9-like enzyme

During the DEAE Sepharose Fast Flow chromatography, DPP activity of bovine testes eluted in 3

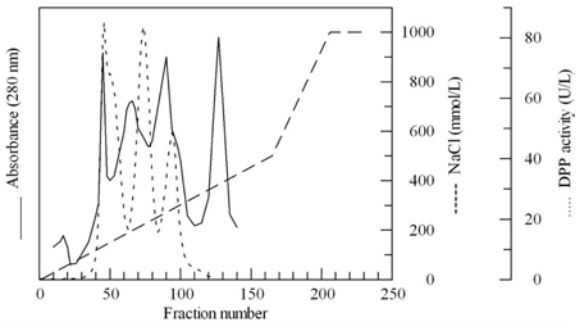

Figure 2. Anion-exchange chromatography on DEAE Sepharose Fast Flow. Bovine testis homogenates were loaded onto the column which was equilibrated with 0.02 M Tris buffer, pH 7.4. Bound protein eluted with a linear gradient in NaCl. …. DPP activity measured by Gly-Pro-pNA; - - - NaCl concentration by continuous measurement of the conductivity; ___ absorbance at 280 nm as an indication for protein content.

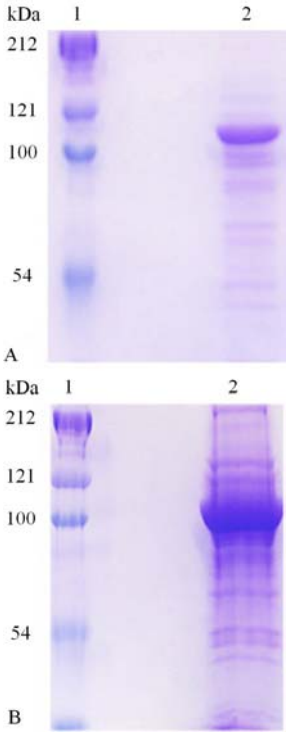

Figure 3. SDS-PAGE analysis of peak 1/DPP9-like enzyme from bovine testes. Samples were resolved by SDS-7.5% PAGE under reducing conditions: active fractions of the HiTrap Q HP chromatography (8 µg, lane 2 figure A) and the Lys-isoindoline affinity gel incubated with SDS-PAGE loading buffer (lane 2 figure B). Molecular Weight Standards are shown in lane 1 of both figures. Proteins were visualized using a Coomassie blue staining.

peaks (Figure 2). The DPP8/9 inhibitor *Allo*-Ile-isoindoline inhibited the DPP activity in peak 1 and 3, whereas the DPPII inhibitor UAMC00039 inhibited the DPP activity in peak 2. The Con A-Sepharose column removed remaining traces of DPPII and DPPIV activity. Further purification of peak 1 from bovine testes was achieved by metal-chelating chromatography, hydrophobic interaction chromatography and ion-exchange chromatography. This procedure resulted in a 1160-fold purification with a yield of 12%, as outlined in Table 1. DPP activity exhibited a high affinity for the inhibitor Lys-isoindoline immobilized onto Sepharose but competitive elution with Lys-isoindoline did not increase the specific DPP activity. SDS-PAGE analysis of two different purification steps of peak 1 are shown in Figure 3. Several incompletely separated protein bands were still present in the latest purification steps precluding mass spectrometrical identification of the individual proteins. The enzyme purified from peak 1 was recognized by binding the polyclonal anti-DPP9 antibody using Western blotting (Figure 4, lane 4). Only the hyperimmune and not the preimmune IgG preparation selectively binds to the purified DPP, which was named from then on 'DPP9-like enzyme'.

The enrichment of peak 3 was achieved by ion-exchange chromatography and the inhibitor-based affinity chromatography. In contrast to the dipeptidyl peptidase activity in peak 1 (DPP9-like enzyme), the active fractions of peak 3 did not bind to the HiTrap nickel-chelating HP column. Another difference was that competitive elution with Lys-isoindoline resulted in a 576-fold enrichment of the specific DPP activity (Table 2). The DPP enriched preparation obtained from peak 3 strongly binds the preimmune and immune IgG preparation and no conclusion could be drawn concerning the presence of DPP9-like enzyme (Figure 4). Also a faint staining of rDPP8 was observed with preimmune and immune IgG preparations.

The DPP9-like enzyme is stable for at least 6 months at -80°C. Storage at 4°C for the same time period resulted in 70% loss of activity. The DPP enriched peak 3 keeps its activity at -80°C during at least 3 months while storage at 4°C this preparation results in 80% loss after 3 months.

4.2. Molecular mass of bovine DPP enriched preparations

The subunit molecular mass of the DPP9-like enzyme was estimated to be approx. 100 kDa based on Western blotting (Figure 4). The enzyme was eluted from the Superdex 200 HR 10/30 size exclusion column with an apparent molecular mass of 164 kDa. DPP enriched peak 3 eluted from the column with an apparent molecular mass of 234 kDa.

4.3. DPP enzymatic staining

After native gel electrophoresis, we could localize DPP activity by derivatisation of the reaction product with Fast Blue B salt. As is documented in Figure 5, human DPPIV, rDPP8, DPP9-like enzyme and DPP enriched peak 3 migrated all on different positions between 128 and 199 kDa, with the kaleidoscope prestained markers as calibrators. With regard to this, it should be noticed that the electrophoresis was carried out under non-denaturing conditions. Both rDPP8 and DPP enriched peak 3 showed

Table 1. Purification of a DPP9-like enzyme from bovine testes

Step	Total protein (mg)	Total activity (U)[1]	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Bovine testis homogenate	22836	164	0.0072	100	1
DEAE Sepharose peak 1	1839	50	0.027	30	4
Con A-Sepharose	1580	53	0.034	32	5
HiTrap Ni Chelating	59	43	0.73	26	101
HiTrap Phenyl	8.7	19	2.2	12	304
HiTrap Q	2.4	20	8.3	12	1160

[1] One unit (U) is defined as the amount of enzyme that cleaves 1 µmol of Gly-Pro-pNA per min under the assay conditions as described in the Materials and Methods section.

Table 2. Enrichment of DPP activity from peak 3 of DEAE Sepharose Fast Flow

Step	Total protein (mg)	Total activity (U)[1]	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Bovine testis homogenate	22836	164	0.0072	100	1
DEAE Sepharose peak 3	1549	20	0.013	12	2
Con A-Sepharose	1207	20	0.017	12	2
HiTrap Ni Chelating	1262	18	0.014	11	2
Phenyl Sepharose CL-4B	316	11	0.035	6.7	5
HiTrap Q	57	4.8	0.084	2.9	12
Lys-Iso[2] wash 1 M NaCl	0.37	0.35	0.95	0.21	132
Lys-Iso[2] elution	0.015	0.062	4.1	0.04	576

[1] One unit (U) is defined as the amount of enzyme that cleaves 1 µmol of Gly-Pro-pNA per min under the assay conditions as described in the Materials and Methods section. [2] Lys-Iso: Lys-isoindoline affinity gel.

more than one activity band which may suggest aggregation of the proteins in solution.

4.4. Effect of detergents and reducing agents on bovine DPP enriched preparations

Figure 6 shows that diluted rDPP8 rapidly loses activity, in contrast to the DPP9-like enzyme and DPP enriched peak 3. The presence of Tween 20 in the dilution buffer stabilized rDPP8 and significantly increased the activity of all the samples.

The effect of the reducing agents DTT and mercaptoethanol on the different enzyme preparations was investigated (Table 3). The presence of the reducing agents increased the activity of the DPP9-like enzyme, while there was no influence on the activity of DPP enriched peak 3 and rDPP8. The increase in DPP activity by reducing agents was not observed for preparations before the Ni-affinity column.

4.5. Kinetic parameters and inhibition characteristics of bovine DPP enriched preparations

The kinetic parameters of bovine DPP enriched preparations for the commercially available dipeptide-derived chromogenic (-pNA) substrates were determined and compared to the K_m values obtained for rDPP8 (Table 4). The DPP9-like enzyme and DPP enriched peak 3 had a K_m value for Gly-Pro-pNA somewhat lower than rDPP8. All 3 enzyme preparations tested had a higher affinity for Ala-Pro-pNA and Arg-Pro-pNA compared to Gly-Pro-pNA. But Arg-Pro-pNA showed a lower V_{max} value compared to Ala-Pro-pNA and Gly-Pro-pNA.

The IC_{50} values of known DPP inhibitors for the DPP9-like enzyme and DPP enriched peak 3 were in the same range as for rDPP8 (Table 5). The peptidase activity of both preparations was inhibited by DFP, proving the serine protease type catalytic mechanism. Both EDTA and aprotinin did not inhibit the activity (Table 3).

4.6. FP-biotin's reactivity with bovine DPP enriched preparations

Experimental evidence for serine type hydrolase mechanism of the bovine DPP enriched preparations was determined using FP-biotin. After SDS-PAGE, we could visualize this enzyme type on blots. As shown in Figure 7, FP-biotin reacted with rDPP8, DPP9-like enzyme and DPP enriched peak 3. This probe showed reactivity with DPP9-like enzyme a little bit lower than with rDPP8, while one thick band of DPP enriched peak 3 was found on the same height as rDPP8 (at 110 kDa).

4.7. pH profiles

The hydrolytic activity of the bovine DPP enriched preparations for Ala-Pro-pNA was assayed in the pH range 4.0-9.2 using a single composite buffer system containing NaCl to maintain comparable ionic strength and identical buffering substances over the entire pH range. Figure 8 illustrates the effect of pH on the enzymatic activity. Both enzyme preparations of DPP9-like enzyme and DPP enriched peak 3 showed an optimum at pH 7.25-7.75. The pH profile of DPP enriched peak 3 had a small peak at pH between 4.7 and 4.9, while DPP9-like enzyme was inactivated completely at acidic pH. At this pH, the activity of DPP enriched peak 3 could not be inhibited by the DPPII inhibitor UAMC00039, proving the absence of DPPII. The lack of inhibition by *Allo*-Ile-isoindoline may suggest the presence of a contaminative dipeptidyl peptidase with an acidic pH optimum.

5. DISCUSSION

In the present study, a dipeptidyl peptidase clearly different from DPPII and DPPIV was purified from bovine testes. It was identified as 'DPP9-like enzyme' by using an anti-DPP9 antibody. A second enriched enzyme preparation also showed DPP8/9-like activity but behaved slightly different in the purification scheme and biochemical characterization.

Table 3. Effect of reducing agents on bovine DPP enriched preparations

Condition	Enzyme preparation	% activity towards control	
			+ EDTA, aprotinin
Control	DPP9-like enzyme	100	184
	DPP enriched peak 3	100	127
	rDPP8	100	132
1 mM DTT	DPP9-like enzyme	169	185
	DPP enriched peak 3	101	130
	rDPP8	108	125
0.125% mercaptoethanol	DPP9-like enzyme	204	234
	DPP enriched peak 3	102	133
	rDPP8	91	122

Samples were diluted in PBS containing 1 mM DTT or 0.125% mercaptoethanol. The Ala-Pro-pNA-cleaving activity was measured in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 with or without the addition of 10 mM EDTA and 14 µg/ml aprotinin. Percent (%) activity towards control is given.

Table 4. Kinetic parameters of bovine DPP enriched preparations

Substrate	rDPP8		DPP9-like enzyme		DPP enriched peak 3	
	K_m	rV_{max}	K_m	rV_{max}	K_m	rV_{max}
Ala-Pro-pNA	167 +/- 18	1	109 +/- 23	1	62.6 +/- 12	1
Gly-Pro-pNA	712 +/- 23	0.95	247 +/- 56	1.03	282 +/- 35	1.03
Arg-Pro-pNA	42 +/- 23	0.48	26 +/- 2.1	0.17	11 +/- 3.1	0.20

Samples were diluted in PBS containing 0.1% Tween 20. Each kinetic experiment was carried out in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20, with the substrates mentioned in the Table. Values for K_m are in µM and rV_{max} is defined as V_{max}X-Pro-pNA/V_{max}Ala-Pro-pNA. The data represent the means +/- S.D. of two to four separate experiments.

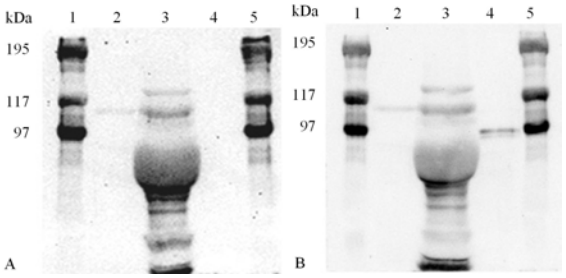

Figure 4. Identification of the DPP9-like enzyme by a polyclonal anti-DPP9 antibody. Samples were resolved by SDS-7.5% PAGE under reducing conditions: rDPP8 (lane 2), DPP enriched peak 3 (lane 3) and DPP9-like enzyme (lane 4). Molecular Weight Standards are shown in lane 1 and 5. Analysis occurred by immunoblotting with preimmune (A) and anti-DPP9 immune rabbit IgG preparation (B). Proteins were visualized using an ECL technique.

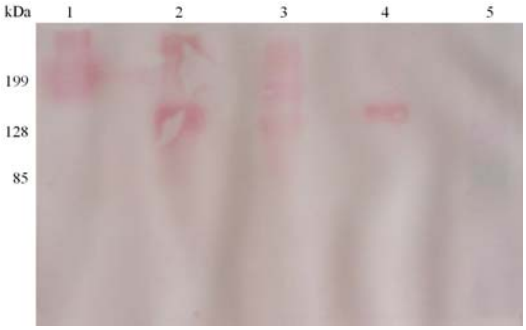

Figure 5. Enzymatic staining of DPP activity after native gelelectrophoresis. Samples diluted in 0.02 M Tris buffer, pH 7.4, containing 0.1% Tween 20 and 1 mM DTT underwent native gelelectrophoresis. Lane 1 contains human DPPIV, lane 2 rDPP8, lane 3 DPP enriched peak 3 and lane 4 DPP9-like enzyme. Molecular Weight Standards are loaded in lane 5.

Previously, only recombinant forms of DPP8 and DPP9 have been purified and characterized. Besides its multiple advantages, recombinant expression always has the limitation that differences due to folding or posttranslational modifications can have an impact on the properties of a protein. The presence of DPP8 and DPP9 in a natural source was demonstrated first at mRNA level (3, 4). Recently, DPP8/9-like activity was shown in human leukocytes and enriched from the cytosol of Jurkat cells (22). Here, we purified and characterized a natural DPP9-like enzyme for the first time. Bovine testes turned out to be a useful source of non DPPII-non DPPIV dipeptidyl peptidases. In the homogenates, the DPPII activity was higher than the DPPIV/8/9 activity, as described earlier by Agrawal *et al.* (27).

The identification of the purified enzyme as a DPP9-like enzyme is supported by immunoblotting and stability data. The kinetic characteristics, the inhibitor profile and biochemical features are comparable with data available for rDPP8 and rDPP9 (7). The polyclonal anti-DPP9 Igs recognized the purified enzyme on Western blotting. Until now we could not observe recognition of the native enzyme by this antibody. The choice of peptides that are candidate immunogens for the development of anti-DPP9 antibodies is rather limited as there is a high degree of sequence conservation within DPP8 and DPP9 among different species. Moreover, the high degree of sequence homology between DPP8 and DPP9 imposed a risk for crossreactivity, especially when peptides are used as immunogens (4). The anti-DPP9 antibody showed no crossreactivity with DPPII, DPPIV (data not shown) and DPP8 (Figure 4).

A second enriched enzyme preparation was obtained from the active fractions of peak 3 from the initial ion-exchange chromatography. These fractions contained about one third of the dipeptidyl peptidase activity of peak

Table 5. Inhibition characteristics of bovine DPP enriched preparations

IC_{50}	rDPP8	DPP9-like enzyme	DPP enriched peak 3
Lys-isoindoline	0.38 +/- 0.02	0.40 +/- 0.01	0.28 +/- 0.01
Allo-Ile-isoindoline	0.02 +/- 0.001	0.08 +/- 0.005	0.03 +/- 0.002
Val-boroPro	0.0024 +/- 0.0001	0.0017 +/- 0.0001	0.0024 +/- 0.00004
AB207	0.03 +/- 0.002	0.05 +/- 0.003	0.04 +/- 0.003
AB192	11 +/- 0.43	18 +/- 0.30	16 +/- 0.61

IC_{50} values were determined at substrate concentrations near the K_m value of rDPP8 diluted in PBS containing 0.1% Tween 20. Each assay was carried out in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 with Ala-Pro-pNA. Values for IC_{50} are in μM. The data represent the means +/- S.D. of two separate experiments.

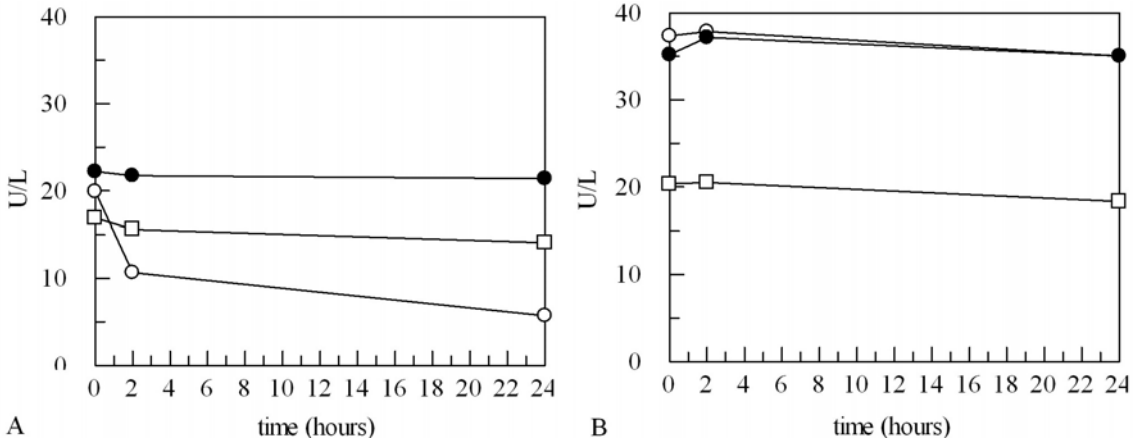

Figure 6. Effect of Tween 20 on the stability of different DPP preparations. Recombinant human DPP8 (○), DPP9-like enzyme (●) and DPP enriched peak 3 (□) were diluted in PBS with (B) or without (A) the addition of 0.1% Tween 20. After 0 min, 2 hours and 1 day at 4°C, DPP activity was assayed in 0.05 M HEPES buffer, pH 7.4, containing 0.1% Tween 20 with Ala-Pro-pNA.

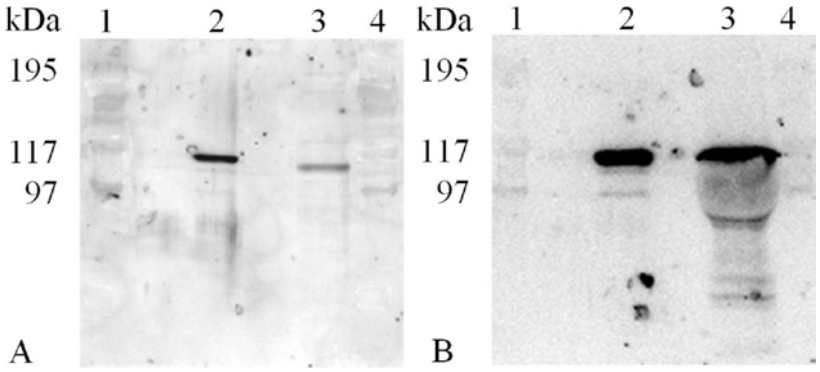

Figure 7. Detection of FP-biotin-reactive proteins from bovine DPP enriched preparations. Samples were incubated with FP-biotin and resolved by SDS-7.5% PAGE under reducing conditions: rDPP8 (lane 2 of the figures), DPP9-like enzyme (lane 3 figure A) and DPP enriched peak 3 (lane 3 figure B). Molecular Weight Standards are shown in lane 1 and 4 of the figures. After electroblotting, proteins were visualized with avidin-HRP by the ECL technique.

1. It is hard to conclude whether we purified 2 different enzymes (eg. DPP9 and DPP8) or whether the enriched enzyme preparation of peak 3 contains the DPP9-like enzyme associated with another protein. The results of the Western blotting, native gelelectrophoresis and the detection with FP-biotin showed that the DPP enriched peak 3 behaved differently from the DPP9-like enzyme.

Using SDS gelelectrophoresis under denaturing conditions, the DPP9-like enzyme had a monomeric molecular mass of approx. 100 kDa, while DPP enriched peak 3 migrated at 110 kDa, as did rDPP8 in this buffer system. It remains open whether this reflects a difference between DPP8 and DPP9 or between the recombinant and natural enzymes. Size exclusion chromatography revealed an apparent molecular mass of 164 kDa for the DPP9-like enzyme. This result is compatible with the occurrence of DPP9 as a dimer in solution as suggested by the experiments of Bjelke *et al.* (7). Recently, Lee *et al.* reported that rDPP8 is predominantly dimeric (8). The higher molecular mass observed for DPP enriched peak 3 (234 kDa) may

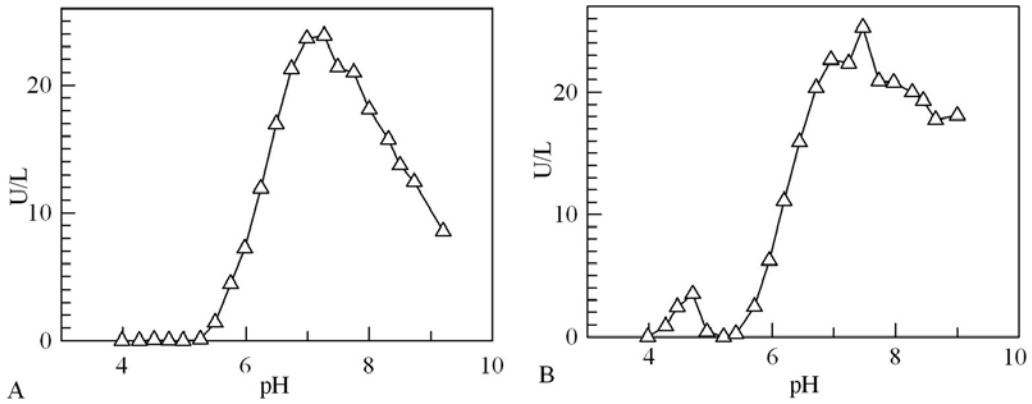

Figure 8. pH profiles of bovine DPP enriched preparations. Measurements were carried out using dilutions for the DPP9-like enzyme (A) and DPP enriched peak 3 (B) as described under Materials and Methods section, with Ala-Pro-pNA as the substrate. Samples used were active fractions of the HiTrap Q HP chromatography (Δ).

indicate binding to another component or the presence of a different peptidase.

DPP8 and DPP9 tend to stick to the walls of the consumable material (eg. tubes, tips). This can be avoided by the addition of the non-ionic detergent Tween 20. During storage at 4°C Tween 20 stabilized rDPP8. Recombinant DPP9 is reported to have a much higher stability than recombinant DPP8 (7). Our findings support the relatively high stability of DPP9. The activity of the purified DPP9-like enzyme increased in the presence of reducing agents. Also EDTA has a more pronounced effect on the DPP9-like enzyme compared to rDPP8 or DPP enriched peak 3. We suggest that metals bind sulfhydryl residues directly or catalyze their oxidation by molecular oxygen in the sample, which cause inactivation of the enzyme. The reducing agents counteract the oxidative effects in the DPP enriched preparations (28, 29). This hypothesis was confirmed by the observation that the effect of reducing agents on the DPP9-like enzyme activity was present in all preparations after the Ni-affinity chromatography and not in the preceding preparations.

The determination of K_m values for the non DPPII-non DPPIV enzymes proved to strongly depend on experimental circumstances much more than is the case for DPPIV. Notwithstanding the experimental variation, most data so far available, including the results of this study, indicate that the basic Arg-Pro-pNA is a preferable substrate in terms of affinity. The turnover rate, in contrast, is much slower compared to Ala-Pro-pNA and Gly-Pro-pNA (7, 8, 20, 30). Because of the similar substrate specificity between DPPIV, DPP8 and DPP9, many DPPIV inhibitors are not highly selective. Inhibitors with an isoindoline at P_1 were selective for DPP8/9 (12, 31). This suggests that these enzymes have a larger S_1 subsite than DPPIV. Here, the Lys-isoindoline column showed a very good affinity for the DPP9-like enzyme. The IC_{50} values of Lys-isoindoline (UAMC00071) for rDPP8 and DPP9-like enzyme were comparable but higher than these of *Allo*-Ile-isoindoline. In this study Val-boroPro was the most potent inhibitor of the purified DPP9-like enzyme and DPP enriched peak 3. This inhibitor showed a highly potent inhibition of all the DPPs, with the highest affinity for DPPIV, DPP8 and DPP9 (12). Val-boroPro, also known as Talabostat or PT-100, is used in several clinical trials in cancer patients (32, 33). Recently, a dual mechanism of action in cancer was suggested for this inhibitor. Besides targeting FAP in the tumor stroma, Talabostat was suggested to stimulate innate and acquired immunity through inhibition of DPP8/9. Further studies using more selective inhibitors will clarify the function of these DPPs. Until now, no inhibitors with a selectivity for DPP8 or DPP9 are available. The purified and enriched preparations were inhibited by DFP and were reactive with FP-biotin. Blotting and visualization of FP-biotin labeled proteins, revealed that the DPP9-like enzyme preparation contained 1 serine type protease. The neutral pH optimum of the DPP9-like enzyme supports its cytoplasmic localization (7, 9).

In conclusion, we have purified a natural DPP9-like enzyme from bovine testes and characterized this serine type peptidase. A second enriched preparation had slightly different biochemical characteristics. Our findings suggest that the purification procedures, the anti-DPP9 antibody and the characterization of natural DPPIV-like enzymes from bovine testes will greatly facilitate further investigations in this family of peptidases and their inhibitors.

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