Assay of Dipeptidyl Peptidase IV in Serum by Fluorometry of 4-Methoxy-2-naphthylamine

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A new fluorometric assay for determining dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) was developed. The synthetic substrate glycyl-L-proline-4-methoxy-2-naphthylamide (20 mmol/L), Tris buffer (50 mmol/L, pH 8.3), and serum (20 μL) are mixed and incubated. The reaction is stopped with citrate (100 mmol/L, pH 4.0) and the released 4-methoxy-2-naphthylamine is measured fluorometrically. The mean value of DPP IV activity in serum for 64 healthy subjects was 58 (SD 16) μmol of 4-methoxy-2-naphthylamine released per liter of serum per minute. The proposed procedure is sensitive, rapid, and accurate and can easily be automated.

Additional Keyphrases: enzyme activity · reference interval screening · cancer · liver disease · hyperthyroidism

Dipeptidyl peptidase IV (DPP IV); postproline dipeptidyl aminopeptidase IV, EC 3.4.14.5 removes N-terminal dipeptides sequentially from polypeptides having unsubstituted N-terminals (1). The penultimate residue must be Pro, Hyp, or Ala, with Pro showing the greatest rate. The N-terminal position may be occupied by several residues. DPP IV cleaves not only substrate P, a potent inflammatory mediator (2, 3), but also casomorphin-5. Recently, some authors evaluated the role of DPP IV in the metabolism of physiologically important peptides (4, 5). Observations that DPP IV activity is altered in various pathological conditions (6–10) generated the need for an accurate, clinically applicable assay. Here we describe a fluorometric method for determining DPP IV activity in human serum. DPP IV catalyzes the cleavage of the fluorogenic substrate, Gly-Pro-4-Me-2-NA, releasing the highly fluorescent 4-Me-2-NA, which can be measured directly. The method is simple, fast, specific, and highly sensitive. It can be automated for routine clinical use.

Materials and Methods

Materials

Serum samples. Blood sampled from 64 blood-bank donors (ages 18–65 y) was allowed to clot at room temperature, centrifuged (2000 × g, 15 min), and the serum was removed and stored at −70 °C until assayed.

Reagents. 4-Methoxy-2-naphthylamine was from Bachem Feinchemikalien, Bubendorf, Switzerland. Gly-Pro-4-Me-2-NA and Gly-Pro-p-nitroanilide tosylate were from Sigma Chemical Co., St. Louis, MO 63178.

All other reagents were of analytical grade from E.

Merck, Darmstadt, F.R.G. In all assays we used de-ionized, distilled water.

Instruments. We measured fluorescence with a Model RF-5000 fluorometer (Shimadzu Corp, Tokyo 160, Japan), with a quartz cuvet (optical path length 0.5 cm). A TE-7 Temptette water bath (Tecam, Cambridge, U.K.) was used for incubations (37 °C ± 0.1 °C). For a colorimetric assay we used a Cobas-Bio centrifugal analyzer (Roche Diagnostic Systems, Montclair, NJ).

Substrate solution. Gly-Pro-4-Me-2-NA, 20 mmol/L, in DMSO. Dissolve 7.37 mg of substrate in 1 mL of DMSO; store at 4 °C. This solution is stable for at least one month.

Incubation buffer. Tris HCl, 50 mmol/L, pH 8.3. Make pH adjustments at room temperature.

"Stopping" solution. Citrate, 100 mmol/L, pH 4.0. Stored refrigerated (4 °C), this solution is stable for at least two months.

Standard solution. The stock solution is 50 mmol/L 4-Me-2-NA in DMSO, prepared by dissolving 8.65 mg 4-Me-2-NA in 1 mL DMSO. Store at −20 °C. The solution is stable for up to a month. Before use, dilute as required with stopping solution.

Procedures

Pipette 0.1 mL of incubation buffer and 20 μL of serum in polypropylene tubes and heat to 37 °C. Start the reaction by adding 10 μL of substrate solution and incubate at 37 °C for 20 min. Stop the reaction by adding 1 mL of stopping solution, then vortex-mix for 30 s. Blanks for each sample are assayed as follows: add stopping solution before starting the reaction instead of afterwards, incubate as described above. Within 1 h after termination of the reaction, measure the fluorescence at 340 and 425 nm (excitation and emission wavelengths, respectively). One unit (U) of DPP IV activity is defined as the enzyme activity that produces 1 μmol of 4-Me-2-NA in 1 min under the conditions described.

Serum DPP IV activity was calculated from the following equation:

\[ \text{Activity, U/L} = \frac{(F \cdot V_t \cdot 1000 \cdot C_w)}{(T \cdot V_s \cdot F_s)} \]

where \( F \) is the fluorescence of the serum sample minus the fluorescence of the serum blank, \( V_t \) the total assay volume (1.13 mL), 1000 the factor for conversion from U/mL to U/L, \( C_w \) the standard concentration (μmol/L), \( F_s \) the fluorescence of the standard minus fluorescence of the solvent, \( T \) the incubation time (20 min), and \( V_s \) the sample volume (20 μL).

Results

Optimum Conditions for Enzyme Assay

Dependence of DPP IV activity on pH. We investigated the pH dependence of DPP IV activity in Tris HCl (pK_a = 8.3), 50 mmol/L, at various pH values (from 6.7 to 9.5). DPP IV exhibits its highest activity over the pH range 8.1–8.9. (Figure 1). pH determinations were made at room temperature.

Determination of Michaelis–Menten constant. DPP IV activity was determined by assaying with substrate concentrations ranging from 0.2 to 350 μmol/L. We calculated \( K_m \),
Sensitivity. We determined the sensitivity of the method by measuring DPP IV activity in diluted serum samples, with the assay conditions mentioned above. We could measure DPP IV activities as low as 5.0 U/L. By prolonging the incubation, lower activities can be detected.

Precision. Within-assay precision was evaluated by analyzing 10 replicate samples of serum in duplicate in a single run. We used three samples with low, medium, and high DPP IV activity. Between-assay precision was calculated after analyzing aliquots of these three samples in 10 different runs over two weeks (Table 1).

Reference interval. For samples from 64 blood-bank donors, the mean value for DPP IV activity was 58 (SD 16) U/L.

Method comparison. For comparison, we measured DPP IV activity by a direct continuous method, in a centrifugal analyzer (Cobas-Bio). This assay is based on the cleavage of the chromogenic substrate Gly-Pro-p-nitroanilide tosylate (11). In a parallel comparison, 30 samples ranging from low to high activity were assayed by both techniques. The results of our fluorometric assay (x, U/L) correlate well with the activities found by colorimetric assay (y, U/L). Least squares linear regression gave the following equation: y = 0.93x + 2.51 (r = 0.971).

Discussion

Several methods have been described for determining DPP IV activity in serum. In most, synthetic substrates that are derivatives of the Gly-Pro dipeptide are used, which provide good affinity and specificity (11). In some methods, colorimetry is used to measure the release of 2-naphthylamine after diazotation. This multi-step method results in lower values for DPP IV activity (22.6 ± 0.9 U/L) (12). The proposed fluorometric procedure assures specificity, precision, and sensitivity. Additional advantages of the method are convenience and economy of time and materials, making it easy to screen large numbers of serum samples. The method correlates well with the colorimetric assay. With the same chromogenic substrate as in that study (11), Fijita et al. (13) obtained a mean value of 55 ± 13 U/L (n = 53), very similar to the mean activity we found with the fluorometric method (58 ± 16 U/L). The increasing interest and probable diagnostic usefulness of DPP IV activity in serum justifies the development of an alternative assay. Significantly higher DPP IV activity was found in serum of patients with hepatobiliary diseases (6). Patients with gastric or pancreatic cancer showed significantly subnormal enzyme activity (9). The increased DPP IV activity in hyperthyroidism may also be of interest, and its cause is to be investigated (14).

Table 1. Precision of the Fluorometric Assay of DPP IV Activity

<table>
<thead>
<tr>
<th>Activity, U/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td>Within-run</td>
<td>64.9</td>
<td>1.3</td>
<td>2.1</td>
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<tr>
<td></td>
<td>39.6</td>
<td>0.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>19.5</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Between-run</td>
<td>73.1</td>
<td>2.6</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>41.9</td>
<td>1.7</td>
<td>4.1</td>
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<tr>
<td></td>
<td>20.8</td>
<td>1.3</td>
<td>6.4</td>
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</tbody>
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n = 10 each.
Further research on DPP IV activity in patients' sera may help to clarify its role in pathology.

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References