Enzymes used for the determination of HbA1C

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Abstract

To develop an enzymatic measurement of HbA1C, two key enzymes, i.e., fructosyl peptide oxidase and Aspergillus protease were characterized. Fructosyl peptide oxidase from Eupenicillium terrenum was a flavoenzyme that could catalyze the oxidation of N-(1-deoxyfructosyl)-Val-His. The enzyme showed high specificity toward α-glycated molecules, therefore it seemed suitable for the HbA1C assay. Since high levels of FPOX expression seemed toxic to host cells, we applied a gene expression system using a bacteriophage vector and achieved high levels of expression in Escherichia coli. Next, we found that Aspergillus protease was able to digest N-(1-deoxyfructosyl)-hexapeptide, a glycated peptide that was released from the β-chain of HbA1C by Glu-C endoproteinase. We showed that the N-(1-deoxyfructosyl)-Val-His released from N-(1-deoxyfructosyl)-hexapeptide by Aspergillus protease could be assayed enzymatically using fructosyl peptide oxidase, therefore these enzymes could be applied to the enzymatic measurement of HbA1C.

Keywords: Hemoglobin A1C; Fructosyl peptide oxidase; Fructosyl amino acid oxidase; Amadoriase; Fructosyl-hexapeptide; Enzymatic measurement of HbA1C; Eupenicillium terrenum

1. Introduction

Glycation is the non-enzymatic reaction by which Amadori compounds are produced from reducing sugars, such as glucose, and an amine. HbA1C is the stable glucose adduct to the N-terminal group of the β-subunit of hemoglobin and has become an important marker for the long-term control of the glycaemic state of diabetic patients with hyperglycemia [1,2].

HbA1C is mostly measured by HPLC and immunoassay. The HPLC method provides good precision but requires a large HPLC device and a long run time. In contrast, large numbers of samples can be measured in a short time with the immunoassay method, however the reproducibility is not good and calibration curve is unstable. Therefore, there is currently great interest in developing an enzymatic assay for HbA1C that could be adapted to an automated analyzer. Basically, the enzymatic assay method for HbA1C consists of two steps: (a) the proteolysis of HbA1C to release glycated amino acid (or glycated peptide) and (b) a reaction by an enzyme (e.g., oxidase or dehydrogenase) that have specificity toward the glycated amino acid (or glycated peptide). Fig. 1(left) shows the IFCC reference method for the measurement of HbA1C [3,4]. In the first step, HbA1C is cleaved into peptides by the endopeptidase Glu-C and in the second step, N-(1-deoxyfructosyl)-Val-His-Leu-Thr-Pro-Glu (Fru-hexapeptide) released from the β-chain of HbA1C is quantified using HPLC and electrospray ionization mass spectrometry or in a two-dimensional approach using HPLC and capillary electrophoresis with UV-detection. We are trying to develop an enzymatic assay that could be adapted to an automated analyzer by modifying the IFCC reference method.

Fructosyl amino acid oxidase (FAOX), or amadoriase, catalyzes the oxidative deglycation of glycated amino acids to produce the corresponding amino acids, glucosone and hydrogen peroxide [5]. FAOXs have been isolated from Corynebacterium sp., Agrobacterium sp., Aspergillus sp., Penicillium sp., Fusarium...
substrate specificity, since we have found there were enzymatic assay system using a FPOX with novel monosodium salt (WST-3). We are developing an Penicillium sp., Ntoward released from Fru-hexapeptide was measured based on FPOX activity. 158 K. Hirokawa et al. / FEMS Microbiology Letters 235 (2004) 157–162
dinitrophenyl)-5-(2,4-disulfophenyl)-2 metalloprotease, FAOX and 2-(iodophenyl)-3-(2,4-enzymatic assay was achieved by using a specific digested by enzymatic assay, the Fru-hexapeptide released by Glu-C was further separated and quantified by HPLC and electrospray mass spectrometry (HPLC-ESI/MS) or by HPLC followed by capillary electrophoresis with UV detection (HPLC-CE). Whereas in the proposed new methods, the Fru-hexapeptide released from the IFCC (International Federation of Clinical Chemis- HbA1c was cleaved into peptides by Glu-C endopeptidase in both methods. In the IFCC (International Federation of Clinical Chemistry) reference method, the Fru-hexapeptide released from the β-chains was separated and quantified by HPLC and electrospray mass spectrometry (HPLC-ESI/MS) or by HPLC followed by capillary electrophoresis with UV detection (HPLC-CE). Whereas in the proposed new enzymatic assay, the Fru-hexapeptide released by Glu-C was further digested by Aspergillus protease. Thereafter, the amount of Fru-ValHis released from Fru-hexapeptide was measured based on FPOX activity.

sp. and Pichia sp. [6–10]. Bacterial FAOXs showed strict specificity toward α-glycated amino acids and had sequence similarity with opine-catabolizing enzymes in Agrobacterium [11]. FAOXs from Aspergillus sp., Penicillium sp. and Fusarium sp. showed specificity toward N-(1-deoxyfructosyl)-N’-Z-Lys, N-(1-deoxyfructosyl)-Val (Fru-Val) and/or fructosyl propylamine [12,9,13]. Recently, we screened a fungal culture collection for fructosyl peptide oxidase (FPOX) using N- (1-deoxyfructosyl)-Val-His (Fru-ValHis) as a substrate [14]. We found that the cell extracts of several genera of fungi showed FPOX activity and then purified and characterized two FPOXs; one from Eupenicillium terrenum ATCC18547 and one from Coniochaeta sp. NISL9330. From the cDNA libraries of E. terrenum and Coniochaeta sp., we cloned cDNA sequences for the FPOXs. Deduced amino acid sequences of the FPOXs showed a high levels of similarity with eu- karyotic FAOXs. A comparison of substrate specificities and amino acid sequences revealed that eu-karyotic FAOXs and FPOXs could be categorized into two groups: (a) enzymes that preferably oxidize α-glycated molecules [9] and (b) enzymes that preferably oxidize α-glycated molecules [15].

Recently, the development of an enzymatic assay for HbA1C was reported [16]. A high precision enzymatic assay was achieved by using a specific metalloprotease, FAOX and 2-(iodophenyl)-3-(2,4-dinitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt (WST-3). We are developing an enzymatic assay system using a FPOX with novel substrate specificity, since we have found there were several proteases that could efficiently release Fru-ValHis from Fru-hexapeptide (data not shown). The principle behind the enzymatic assay we are developing is described in Fig. 1(right). HbA1C is cleaved by Glu-C yielding Fru-hexapeptide according to the IFCC reference method. This procedure is followed by an additional proteolysis that releases Fru-ValHis, since Fru-ValHis can be measured with a FPOX reaction.

In this study, we demonstrate that Fru-ValHis is ef- ficiently liberated from Fru-hexapeptide by Aspergillus protease and becomes a substrate for FPOX. The high-level production of FPOX with a bacteriophage gene expression system for industrial use is also investigated.

2. Materials and methods

2.1. Materials

N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt (TOOS) was purchased from Dojindo Laboratories, Japan, and horseradish peroxidase from Kikkoman, Japan. Fru-hexapeptide was purchased from Peptide Institute Inc., Japan. Fru-ValHis and other fructosyl amino acids were prepared as described previously [5].

2.2. Assay of FPOX activity

FPOX activity was measured spectrophotometrically at 37 °C with the peroxidase-coupled reaction system, as described previously [11]. The standard reaction mixtures contained 0.1 M potassium phosphate, pH 8.0, 0.5 mM TOOS, 900 U/l of peroxidase, 0.45 mM 4-aminopyridine and 20 mM Fru-ValHis in a final volume of 3 ml. One unit of enzyme activity was defined as the amount of enzyme that produced 0.5 μmol of quin- oneimine dye per minute at 37 °C.

2.3. Construction of FPOX expression cell using a ‘Sleeper’ bacteriophage vector

FPOX cDNA from E. terrenum was subeloned into a ‘Sleeper’ vector constructed from bacteriophage λ and φ80 [17,18]. The recombinant phage DNA was introduced into Escherichia coli by the method of in vitro packaging to obtain the FPOX gene lysogen, “FPOX- Sleeper” [19].

2.4. Preparation of cell extracts and purification of FPOX

FPOX-Sleeper was grown aerobically at 32 °C in 6 l of Luria broth (1.0 g of polypeptone, 0.5 g of yeast extract and 0.5 g of NaCl in 100 ml, pH 7.4). At the late log phase, the culture was shifted to 42 °C for
3. Results and discussion

3.1. Expression of FPOX cDNA in E. coli

FPOX was expressed as an active form when the plasmid carrying FPOX cDNA from *E. terrenum* was introduced into *E. coli* JM109 [15]. The level of FPOX activity in the cell extract was not so high (0.01 U/ml), probably because a high level of FPOX expression was toxic to the host cell, *E. coli* [20]. To increase the FPOX gene expression, we applied an expression system using a bacteriophage ‘Sleeper’ vector [17,18]. In the ‘Sleeper’ system, the FPOX gene expression could be strictly repressed before heat induction; therefore it seemed suitable for expressing a heterogeneous gene whose product was toxic to the host cell. The FPOX activity in the cell extract of FPOX-Sleeper was 0.17 U/ml, which was 66 times higher than that in the cell extract of the original strain, *E. terrenum*.

3.2. Properties of recombinant FPOX

The FPOX from FPOX-Sleeper was purified from the cell extract as described in Section 2. The purified preparations showed single bands on SDS-PAGE, indicating an apparent homogeneity of the protein. The purified FPOX had a specific activity of 5.4 U/mg of protein.

2.5. Identification of proteolysis and deglycation products

Fru-hexapeptide, Fru-ValHis and valyl-histidine (ValHis) were measured by HPLC on an Amide-80 column (ValHis) were measured by HPLC on an Amide-80 2.5 M). Identification of proteolysis and deglycation products

2.6. Enzymatic measurement of glycated peptide

Fru-hexapeptide dissolved in water at several concentrations was used as the standard solution. Five microliters of the standard solution and 5 µl of *Aspergillus oryzae* protease (20 µg/ml; Kikkoman, Japan) in 50 mM potassium phosphate buffer (pH 6.5) were incubated at 37 °C for 2 h. After heat inactivation (90 °C, 5 min), 140 µl of reaction mixture was added and incubation was continued at 30 °C for 20 min, then the absorbance of the solution at 555 nm was measured. The reaction mixture contained 0.1 M potassium phosphate, pH 8.0, 0.5 mM TOOS, 900 U/l of peroxidase, 0.45 mM 4-aminoantipyrine and 0.1 U/ml of FPOX in a final volume of 150 µl.
In its pH and temperature properties, the recombinant protein was indistinguishable from the native protein purified from *E. terrenum*. Apparent $K_m$ values of FPOX were 2.76 mM for Fru-ValHis and 0.318 mM for Fru-Val. The specificity toward Ne-(1-deoxyfructosyl)-Lys (eFru-Lys) was quite low compared to Fru-Val and Fru-ValHis. Therefore, the FPOX seemed suitable for the assay of HbA1C, since internal Lys residues of blood proteins are glycated when the glucose level is high, and so eFru-Lys becomes a major contaminant of the HbA1C assay. The enzyme activity was inhibited considerably by Mn$^{2+}$ and Co$^{2+}$, and partially by other metal ions (Table 1). The enzyme was strongly inhibited by phenylhydrazine. This result indicated that sulfhydryl and carbonyl groups exist at the catalytic site of the enzyme and are important for the enzyme reaction.

3.3. HPLC analysis of proteolysis of Fru-hexapeptide and deglycation of Fru-ValHis

HbA1C is measured with the β-N-terminal hexapeptide (Fru-hexapeptide), which is released by enzymatic

Table 1

<table>
<thead>
<tr>
<th>Addition (10 mM)</th>
<th>Activity (%)</th>
<th>Addition (1.0 mM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>None</td>
<td>100</td>
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<tr>
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<td>93</td>
<td>FeCl$_3$</td>
<td>79</td>
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<tr>
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<td>95</td>
<td>Al$_2$(SO$_4$)$_3$</td>
<td>94</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>89</td>
<td>ZnCl$_2$</td>
<td>89</td>
</tr>
<tr>
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<tr>
<td>NaN$_3$</td>
<td>96</td>
<td>BaCl$_2$</td>
<td>99</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>0</td>
<td>CuSO$_4$</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme assay was performed without additions or with different compounds (10 mM or 1.0 mM final concentrations) added to the reaction mixture as described under Section 2.

Activity is expressed as a percentage of the activity without additions.
cleavage of the intact glycated hemoglobin molecule with the endoproteinase Glu-C, using HPLC-ESI/MS or HPLC-CE ([3,4], Fig. 1(left)). As shown in Fig. 1(right), the development of a simple enzymatic assay for HbA1C could be achieved by applying a protease that liberates the substrate for FPOX. We screened a number of proteases using Fru-hexapeptide as substrate and found the Aspergillus protease to be one of the most effective for liberating the substrate for FPOX [15]. To confirm the proteolytic activity of the Aspergillus protease, the reaction substrate and product were analyzed by HPLC (Fig. 4). After treatment with the protease for 3 h, the Fru-hexapeptide peak disappeared and then a Fru-ValHis peak appeared (Fig. 4(a) and (b)). Furthermore, the proteolytic sample of Fru-hexapeptide was used as the substrate for the FPOX reaction. After treatment with FPOX for 30 min, the Fru-ValHis peak disappeared and a ValHis peak appeared (Fig. 4(c)). These results indicated that (a) Fru-ValHis was released on incubation of Fru-hexapeptide with the protease and (b) the Fru-ValHis was enzymatically deglycated by FPOX.

3.4. Time-dependent release of Fru-ValHis from Fru-hexapeptide

Since Fru-ValHis could be easily assayed with the FPOX reaction, a time-course experiment on the release of Fru-ValHis from Fru-hexapeptide by Aspergillus protease was performed (Fig. 5). The amount of Fru-ValHis released increased in a time-dependent manner and reached a maximum over the 120 min of incubation. No absorbance was detected when a bacterial FAOX that had no activity toward Fru-ValHis was used (data not shown), indicating that not Fru-Val but Fru-ValHis was released by the protease treatment.

3.5. Enzymatic measurement of glycated hexapeptide

For the enzymatic determination of Fru-hexapeptide, protease-treated Fru-hexapeptide was used as substrate for the FPOX reaction. As shown in Fig. 6, a linear relationship was observed between the concentrations of protease-treated Fru-hexapeptide and the absorbance for FPOX reactions. To estimate the proteolytic efficiency of the protease, the enzyme assay was performed with varying concentrations of Fru-ValHis. At each substrate concentration, the absorbance for Fru-hexapeptide was 80% of that for Fru-ValHis, indicating that about 80% of Fru-hexapeptide was digested by the protease releasing Fru-ValHis. These results indicated that FPOX is applicable for determining the amount of Fru-hexapeptide that is supposed to be liberated from HbA1C by Glu-C endoproteinase [3,4].

3.6. General implications

Previously, we cloned FPOX cDNA from E. terremun and expressed the gene in E. coli, however the level of expression was low when a plasmid vector was used. In the present study, we achieved a high level of expression using a bacteriophage expression system. Recombinant
FPOX showed high specificity toward α-glycated molecules and low specificity toward ε-Fru-Lys. Therefore, the FPOX seemed suitable for the assay of HbA1C, because internal Lys residues of proteins are glycated when the glucose level is high, and so ε-Fru-Lys becomes a major contaminant of the assay system. As FPOX acts mainly on Fru-ValHis even in the presence of major contaminant of the assay system, it should provide for a precise assay of HbA1C. We showed that Aspergillus protease was able to digest fructosyl-hexapeptide, a glycated peptide that was released from HbA1C by Glu-C endoproteinase. As shown in Fig. 6, the Fru-ValHis that was released from fructosyl-hexapeptide by Aspergillus protease could be assayed enzymatically using FPOX, therefore these enzymes were applicable to the enzymatic measurement of HbA1C. From these results, both enzymes, i.e., FPOX and Aspergillus protease are supposed useful for an enzymatic assay of HbA1C.

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References