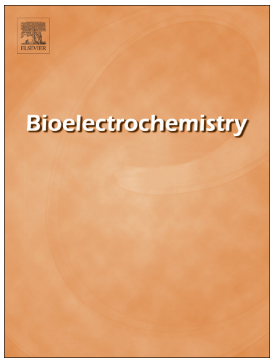

Discovery of a novel quinohemoprotein from a eukaryote and its application in electrochemical devices

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PII: S1567-5394(19)30246-4

DOI: https://doi.org/10.1016/j.bioelechem.2019.107372

Reference: BIOJEC 107372

To appear in: *Bioelectrochemistry*

Received date: 16 April 2019

Revised date: 4 September 2019

Accepted date: 4 September 2019

Please cite this article as: K. Takeda, K. Igarashi, M. Yoshida, et al., Discovery of a novel quinohemoprotein from a eukaryote and its application in electrochemical devices, *Bioelectrochemistry* (2019), https://doi.org/10.1016/j.bioelechem.2019.107372

Discovery of a novel quinohemoprotein from a eukaryote and its application in electrochemical devices

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Abstract:

Pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase is one of the extensively studied sugar-oxidizing enzymes used as a biocatalyst for biosensors and biofuel cells. A novel pyranose dehydrogenase (*Cc*PDH) derived from the basidiomycete *Coprinopsis cinerea* is the first discovered eukaryotic PQQ-dependent enzyme. This enzyme carries a *b*-type cytochrome domain that is homologous to the cytochrome domain of cellobiose dehydrogenase (CDH); thus, *Cc*PDH is a quinohemoprotein. *Cc*PDH catalyzes the oxidation of various aldose sugars and shows significant activity toward the reverse-chair conformation of pyranoses. Interdomain electron transfer occurs in *Cc*PDH similar to CDH, from the PQQ cofactor in the catalytic domain to the heme *b* in the cytochrome domain. This enzyme is able to direct electrical communication with electrodes, without artificial electron mediators, thus allowing direct electron transfer (DET)-type bioelectrocatalysis. In this review, we briefly describe recent progress in research on the biochemical discovery of *Cc*PDH and the development of (bio)electrochemical applications (an amperometric biosensor) based on DET reactions.

Keywords

pyrroloquinoline quinone, heme, quinohemoprotein, direct electron transfer, biosensor, biofuel cell

Highlights

- The electron transfer reaction of *Cc*PDH is summarized.
- A biosensor of L-fucose, a cancer marker, was prepared using *Cc*PDH.
- *Cc*PDH is also useful as an anode catalyst for DET-type biofuel cells.

1. Introduction

Pyrroloquinoline quinone (PQQ) was extracted and identified as a coenzyme from methanol dehydrogenase of methylotrophs [1, 2]. Enzymes having PQQ as a coenzyme (PQQ-dependent quinoproteins) have been widely distributed, such as methanol dehydrogenase, glucose dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, glycerol dehydrogenase, sorbitol dehydrogenase, and so on. All of these catalyze mainly the oxidation of the primary or secondary hydroxyl group in the substrate. They have a common basic structure with propeller superbarrel fold made up of eight- or six-bladed, which each blade consisted four-stranded antiparallel beta-sheet and arranged toroidally around a central axis [3-5]. For bioelectrochemical applications, advantages of the PQQ-dependent quinoproteins are that dioxygen is not involved in the catalytic reaction and the cofactor PQQ is relatively strongly bound to the enzyme. Therefore, unlike NAD(P)-dependent dehydrogenases, the addition of the cofactor to the application system is not necessary. The most emphasized feature of PQQ-dependent quinoproteins is their broad substrate specificity, especially when compared to FAD-dependent enzymes. While this is disadvantageous for sensing applications, it can be advantageous for applications such as bioreactors and biofuel cells in the sense that PQQ-dependent quinoproteins can oxidize a variety of substrates. Minteer and co-workers reported that glycerol or glucose can be completely oxidized to carbon dioxide with a simple three or six enzymes cascade thanks to utilizing the lack of specificity of PQQ-dependent quinoproteins [6, 7]. Our previous study demonstrated that a PQQ-dependent alcohol dehydrogenase from *Pseudomonas putida* KT2440 (*Pp*ADH) catalyzes the oxidation of alcohol and that it can also further oxidize aldehydes. It means that the two-step oxidation of ethanol to acetate can be catalyzed on the anode with only one enzyme, *Pp*ADH [8].

All PQQ-dependent quinoproteins reported so far are of prokaryotic origin and have not been reported in eukaryotes. Recently, our research group found a gene (*ccpdh*) that has very low homology to the amino acid sequence of the known PQQ-dependent quinoproteins in the whole genome sequence of the basidiomycete *Coprinopsis cinerea*, which is a eukaryote [9]. This gene has a unique putative PQQ domain sandwiched between the N-terminal cytochrome domain and the C-terminal carbohydrate-binding module (CBM) (Fig. 1). The recombinant protein expressed by introducing this gene using *Pichia pastoris* as a host showed carbohydrate oxidation activity in the presence of PQQ and was thus named *Cc*SDH (*C. cinerea* sugar dehydrogenase) [9]; it was later renamed *Cc*PDH (*C. cinerea* pyranose dehydrogenase; EC 1.1.2.B5) [10]. These findings led to the discovery of the first PQQ enzyme derived from a eukaryote.

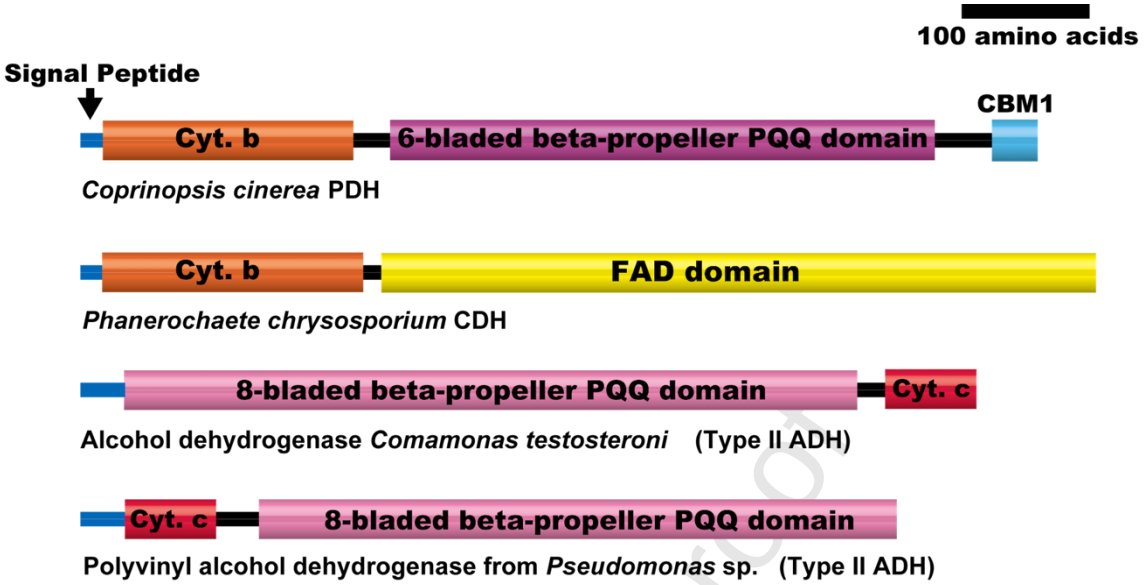


Fig. 1. Domain structure of *Cc*PDH and CDH from *P. chrysosporium* and bacterial PQQ quinohemoproteins. Abbreviations: Cyt. b, cytochrome *b* domain; CBM1, family 1 carbohydrate-binding module; Cyt. c, cytochrome *c* domain. Reproduced from Ref. [25].

Quinoproteins with a heme domain are collectively referred to as quinohemoproteins. PQQ-dependent quinohemoproteins that have been reported so far are alcohol dehydrogenases derived from bacteria that have the cytochrome *c* domain [5, 11] (Fig. 1). The newly discovered *Cc*PDH has a cytochrome *b* domain and can be considered a new quinohemoprotein. Since these quinohemoproteins can perform a direct electron transfer (DET) reaction with an electrode [12-16], a very simple enzyme electrode reaction system can be constructed by using these enzymes. Our group is also working on a practical application of this novel PQQ-dependent dehydrogenase as an electrocatalyst for a DET-type amperometric sensor. In this review, we describe the enzymatic functions, the direct electron transfer reaction of *Cc*PDH with the electrode, and the development of electrochemical devices based on these phenomena.

2. *Cc*PDH: PQQ-dependent pyranose dehydrogenase from a basidiomycete

The PQQ domain of *Cc*PDH strongly binds to PQQ at a ratio of 1: 1 (K_d = 1.1 nM) despite the low homology (approximately 15-20%) of the primary amino acid sequence with the known PQQ enzymes [9]. Recently, our group succeeded in crystallization of the holo PQQ domain. The X-ray crystallographic analysis by our group revealed the three-dimensional structure in which PQQ is bound to the active center [17]. It has a six-bladed superbarrel structure, which consists of antiparallel four-stranded ß-sheets,

common to PQQ glucose dehydrogenases, and PQQ is located at a short distance from the surface (Fig. 2A). In *Cc*PDH, one of the Arg residues (R273) bound to PQQ is not conserved in the sequence alignment of known PQQ glucose dehydrogenases; however, this Arg residue was structurally conserved (Fig. 2B). This amino acid residue was identified as a PQQ-binding amino acid residue characteristic for basidiomycetes.

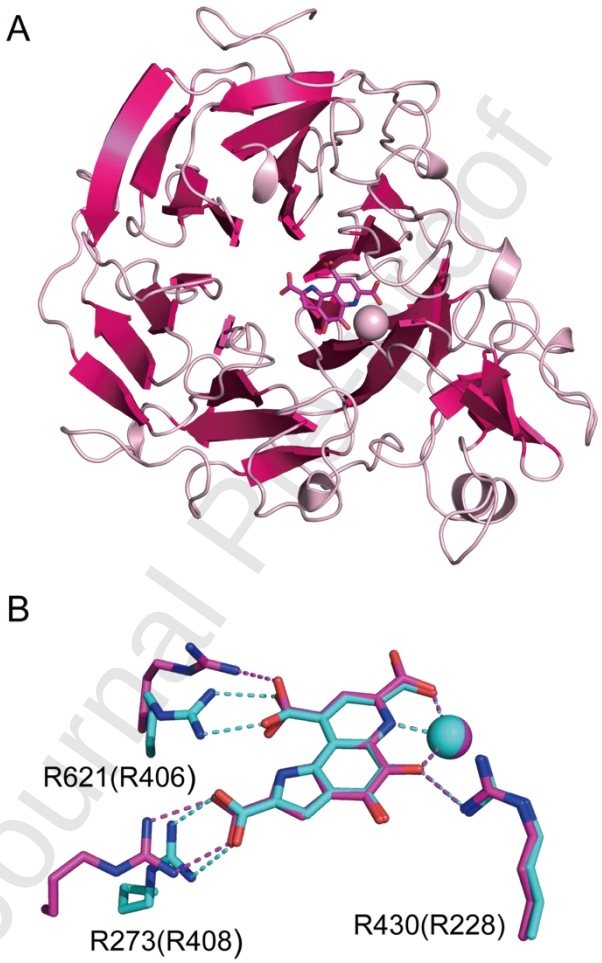


Fig. 2. X-ray crystal structure of the PQQ domain *Cc*PDH (PDBID=6JWF). (A) Overall structure of the PQQ domain of *Cc*PDH. ß-Strands are shown as *magenta* arrows. (B) Stick representation of arginine residues binding PQQ in the active site of *Cc*PDH (*magenta*) and soluble glucose dehydrogenase from *Acinetobacter calcoaceticus* (sGDH, *cyan*). Residue labels in *parentheses* represent the numbering for sGDH (PDBID=1CQ1), PQQ and calcium ion are shown as stick and sphere models, respectively.

The theoretical molecular weight and pI of the full-length *Cc*PDH (without signal

peptide) are 77 kDa and 7.7, respectively. This pI value was calculated by using Compute pI / Mw tool on the ExPASy server [18]. The recombinant enzyme expressed in *P. pastoris* has a molecular weight of 98±8 kDa on SDS-PAGE due to *N*- and *O*-glycosylation. *Cc*PDH has no activity toward common monosaccharides, such as D-glucose, D-fructose, and cellobiose, which are the degradation products of cellulose. On the other hand, *Cc*PDH can oxidize rare sugars including D-glucosone (2-keto-D-glucose), L-fucose (6-deoxy-L-galactose), D-arabinose and L-galactose. For example, the K_m for L-fucose was 24.8 mM, and the k_{cat} was 56.4 s^{-1} [9]. Since these pyranoses are in the reverse-chair conformation ($^1C_4$), kinetic analysis was performed to analyze the three-dimensional structure of the sugars that can be a substrate. *Cc*PDH selectively reacts with pyranose in the reverse-chair conformation with equatorial OH groups at the C-2 and C-3 positions and an axial group at the C-4 position. In particular, the orientation of the OH groups at the C-2 and C-4 positions seems to be important [10]. Identification of the oxidized product clearly indicated that this enzyme oxidizes the OH group at the C-1 position of the pyranose moiety [19]. The K_m values of *Cc*PDH for the monosaccharides can be high compared to the K_m values of the common enzymes; however, the values are similar to those of bacterial PQQ dehydrogenases reported so far. Even the soluble PQQ glucose dehydrogenase from *Acinetobacter calcoaceticus* has a K_m value of 25 mM for its physiological substrate, D-glucose [20]. In general, PQQ enzymes are characterized by high stereospecificity but low substrate specificity, and they react with numerous substrates [21, 22]. Similar features were observed in *Cc*PDH.

The UV-vis absorption spectrum and resonance Raman spectrum of the heme domain of *Cc*PDH indicate that the heme of *Cc*PDH has Met and His as ligands similar to the heme of cellobiose dehydrogenase (CDH; EC 1.1.99.18); the oxidized and reduced forms have six-coordinated low-spin states [10, 23, 24]. *Cc*PDH has strong adsorption on cellulose due to CBM [10]. It is suggested that this enzyme localizes and acts on the surface of cellulose outside the cell. Furthermore, Vicent et al. showed that *Cc*PDH is an electron donor of lytic polysaccharide monooxygenase (LPMO) that oxidatively degrades cellulose [19]. Thus, the physiological role of *Cc*PDH apparently involves plant cell wall degradation by functioning as an auxiliary enzyme for cellulolytic enzymes.

A number of genes in numerous fungal genomes are homologous to the PQQ domain of *Cc*PDH with four types of domain organizations: single PQQ domain (majority), PQQ domain - CBM, cytochrome domain - PQQ domain, and cytochrome domain - PQQ domain - CBM [25]. In addition to fungi, BLAST search revealed that other eukaryotes, such as mold, amoeba and a number of bacteria, have genes with high

homology to the PQQ domain of *Cc*PDH. Molecular phylogenetic analysis identified the gene family containing *Cc*PDH that forms a group different from the known PQQ-dependent dehydrogenase family [9]. This indicates the presence of a completely new family of PQQ enzymes that has not been known so far. One of the new family members, a gene derived from *Pseudomonas aureofaciens*, was recombinantly expressed to examine its function and was found to be a PQQ-dependent dehydrogenase which oxidizes D-glucosone as a substrate [26]. Thus, the fact that PQQ enzymes recently found in eukaryotes cannot be detected by homology search using the known PQQ enzymes suggests the presence of other unknown PQQ enzymes in other species. The importance of research on PQQ enzymes is expected to increase in the future.

3. Electron transfer pathway and direct electron transfer reaction of *Cc*PDH

*Cc*PDH has been discovered by homology search using the cytochrome domain of CDH in a filamentous fungus (Fig. 1), indicating that the amino acid sequence of the cytochrome domain on the N-terminal region of *Cc*PDH corresponds to the sequence of the cytochrome domain of CDH [9]. The overall structure of the heme domain is rich in ß-sheets similar to that of the CDH heme domain with an RMSD value of 1.3 Å (manuscript in preparation). However, the structures specifically differ in the amino acid residue that is considered to be involved in the intramolecular electron transfer around the heme. Interestingly, the surface charge around the heme is negative in CDH from *Phanerochaete chrysosporium*, whereas the charge is positive in *Cc*PDH (Fig. 3). These differences suggest that the optimum conditions for intramolecular electron transfer in *Cc*PDH and that in CDH are different [27].

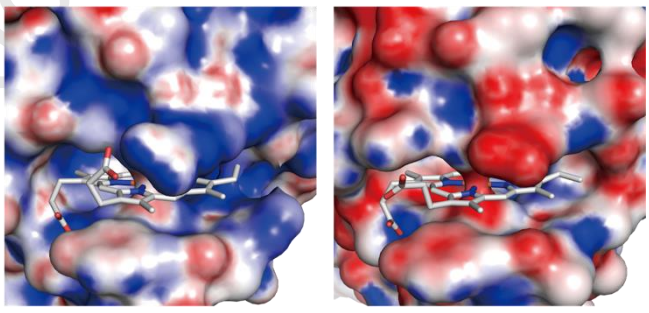


Fig. 3. Comparison of electrostatic surface charge of the heme binding pocket in *Cc*PDH (left, PDBID=6JT6) and *Pc*CDH (right, PDBID=1D7D). Heme is shown as white *stick* model. Positively charged regions are *blue* and negatively charged regions are *red*. The electrostatic surface potential is displayed using by the PyMol APBS plugin and the color coded from red (-10 kT) to blue (+10 kT). Reproduced from Ref. [17].

The addition of L-fucose, one of the substrates, to the full-length *Cc*PDH solution gives the UV-vis absorption spectrum of the reduced form of the heme *b* [10]. This is evidence of the intramolecular electron transfer from the PQQ domain to the cytochrome *b* domain. The heme *b* in CDH is exposed to the molecular surface, enabling direct electron transfer reaction with various electrodes [16, 28-31]. Therefore, a possibility of a direct electron transfer reaction in *Cc*PDH was examined. An enzyme electrode was fabricated and composed of full length *Cc*PDH immobilized on a carbon electrodes, and cyclic voltammetry measurements were performed (Fig. 4). As a result, direct redox currents were observed between the enzyme and the electrode at approximately the same redox potential as that of the heme *b* of CDH ($E^{0'}$ = 130 mV vs. NHE at pH 7.0) [9]. It was found that *Cc*PDH is an enzyme capable of direct electron transfer reaction with the electrode *via* the heme moiety of the molecule. Enzymes with an electron transfer site such as quinohemoproteins are very useful as electrocatalysts.

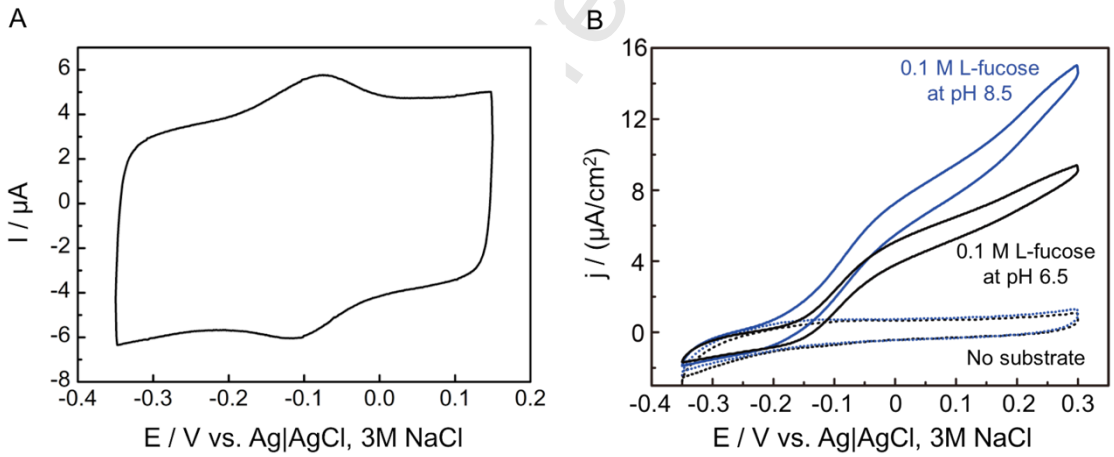


Fig 4. (A) Cyclic voltammogram of *Cc*PDH immobilized on a plastic-formed carbon electrode modified with carbon nanoparticles. The voltammogram was obtained in 100 mM HEPES buffer (pH 7.0) at a scan rate of 20 mV s^{-1}. Adapted from Ref [10]. (B) Cyclic voltammograms of the *Cc*PDH modified glassy carbon (GC) electrode at pH 6.5 (black line) and 8.5 (blue line) in the absence (dashed line) and presence (solid line) of 100 mM L-fucose, at a scan rate of 5 mV s^{-1}. Adapted from Ref [27].

In the natural electron transfer reaction of *Cc*PDH, interdomain electron transfer proceeds from PQQ reduced by oxidation of a substrate to the heme of the cytochrome domain; it is believed that the electron is transferred from the heme to LPMO [19]. However, depending on the electron acceptor used, it is possible to receive electrons

directly from the PQQ domain bypassing the heme [27]. The optimum pH for the oxidation reaction of L-fucose is pH 6, and in this case, the electron is directly transferred from the PQQ domain to the electron acceptor, while at pH 8.5, the electron is transferred via the cytochrome domain (Fig. 5). This phenomenon indicates that inter-domain electron transfer (IET) is strongly dependent on pH. Furthermore, it indicates that, in the pH range from 6 to 8.5, the IET between PQQ and heme is slower than the oxidation reaction of the substrate or the electron transfer reaction between the heme and the electron acceptor and that this is a rate-limiting step. The results obtained by immobilization on the electrode identified the conditions for electrochemical determination of the redox potentials of the cytochrome and PQQ domains; thus, the redox potentials at each pH value of PQQ and heme in the enzyme were determined (Takeda et al., unpublished results). It became clear that the potential difference between the moieties varies between pH 6.0 and 8.5; it was presumed that this pH-dependent change in potential differences influences the intramolecular electron transfer reaction.

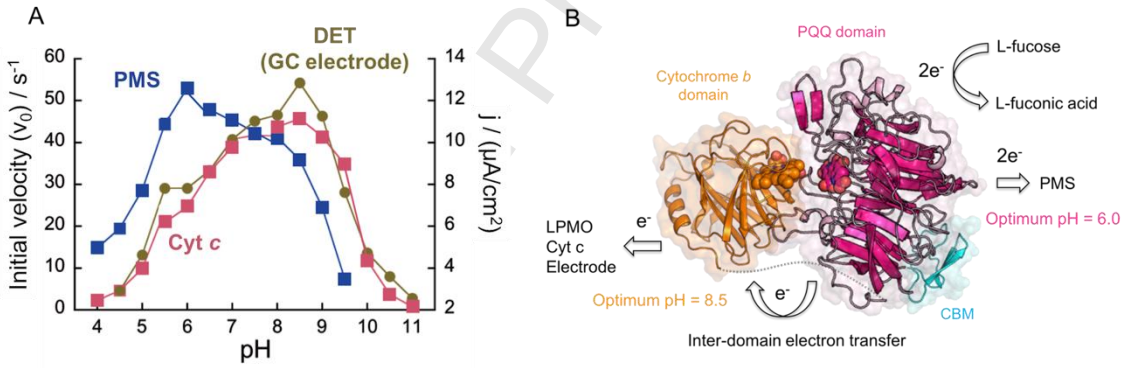


Fig. 5. (A) Comparison of pH dependence of the catalytic activities and the DET based bioelectrocatalytic current. The catalytic activities *Cc*PDH using phenazinium methylsulfate (PMS; blue line) and cytochrome c (Cyt *c*; magenta line) with L-fucose as the substrate. The DET based bioelectrocatalytic current of the *Cc*PDH-modified GC electrode at 0.3 V vs. Ag|AgCl, 3M NaCl in the presence of L-fucose. Reproduced from Ref. [27]. (B) Schematic representation of the electron transfer pathways from the substrate through *Cc*PDH to various electron acceptors. Electron pathways are shown as arrows. Pyranose oxidation takes place at the PQQ domain followed by inter-domain electron transfer (IET) to the cytochrome *b* domain. Subsequent to the reduction of the heme, the electron is delivered to lytic polysaccharide monooxygenase (LPMO), Cyt *c*, or an electrode via the cytochrome *b* domain. Alternatively, PMS, an artificial electron acceptor, can be reduced directly by $PQQH_2$ in the PQQ domain.

4. Development of a biosensor to detect urinary L-fucose

One of the substrates of *Cc*PDH, L-fucose (Fig. 6), is a constituent of the polysaccharides included in the cell wall of brown algae such as kelp [32-34].

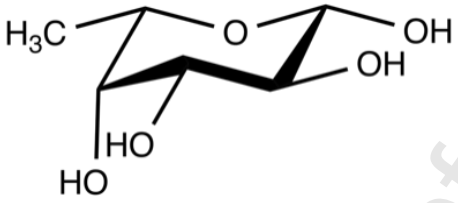


Fig. 6. L-Fucose (the reverse-chair conformation of L-fucose, L-fucopyranose)

In animals and humans, L-fucose binds to the nonreducing end of the complex sugar chain on the cell surface and is considered to be an important sugar for intercellular communication and immune response [35-37]. The details of L-fucose metabolic pathway in humans remain unclear; however, it is known as a tumor marker because its concentration is increased in the urine and serum of certain cancer patients [38-42]. Sakai et al. reported that free L-fucose concentration in urine in cancer patients was higher than that in healthy people [43]. Urine volume is influenced by food intake, fluid intake and dehydration. The concentration of most urinary components greatly varies depending on urine volume. Therefore, the concentrations of urinary components are routinely corrected versus the urinary creatinine concentration. Sakai et al. measured the concentration of free L-fucose in urine in 260 healthy subjects including men and women, and the reference value was 148 μmol / g of creatinine. The cutoff value dividing the normal range from the abnormal values was determined as the reference value ± 2 SD (standard deviation), and it was 215 μmol / g of creatinine. When free urinary L-fucose concentration was measured in 366 cancer patients, the free urinary L-fucose concentration in 206 patients was higher than the cutoff value [43].

Conventional methods for detecting L-fucose in the urine include chromatography and HPLC combined with spectroscopic or fluorescent detection. However, these methods have problems including long measurement time, use of expensive equipment, and required pretreatment for removal of urinary impurities. On the other hand, generally, an electrochemical measurement has the advantages of low cost, short measurement time and easy operation. Therefore, it is expected that the measurement of L-fucose can be performed by using a simple electrochemical measurement method. The electrochemical measurement methods using NADH-dependent fucose

dehydrogenase (EC 1.1.1.122) have been reported [44]. However, this method requires a mediator to assist with reoxidation of reduced NADH at the electrode; moreover, it is difficult to avoid the effects of interfering substances present in the urine, such as uric acid, dopamine and ascorbic acid. Therefore, an amperometric L-fucose sensor of DET type was prepared using *Cc*PDH. A glassy carbon electrode modified with carbon nanoparticles (CNP) was immersed in 10 µM *Cc*PDH solution containing PQQ for 6 hours. The responsiveness to L-fucose was examined by chronoamperometry measurement (applied potential +0.1 V vs. Ag / AgCl (3 M NaCl)) using the prepared electrode as a working electrode. In response to the stepwise addition of L-fucose, the current density rapidly increased (Fig. 6). The current density obtained in the L-fucose concentration range from 0.04 mM to 2.1 mM with a cutoff value of 0.17 mM showed a good linearity with a correlation coefficient r^2 = 0.9996 (Fig. 7). The cutoff value of L-fucose in a 60 kg adult male was assumed to be 0.17 mM (creatinine excretion was 20 mg / kg and urine excretion was 1.5 L / day). The sensor sensitivity was 4.54 mA cm^{-2} mM^{-1} and the detection limit was calculated to be 0.6 µM at a signal-noise ratio S / N = 3. Thus, the sensitivity is sufficiently high for a detection sensor of free L-fucose in urine (Takeda et al., unpublished results).

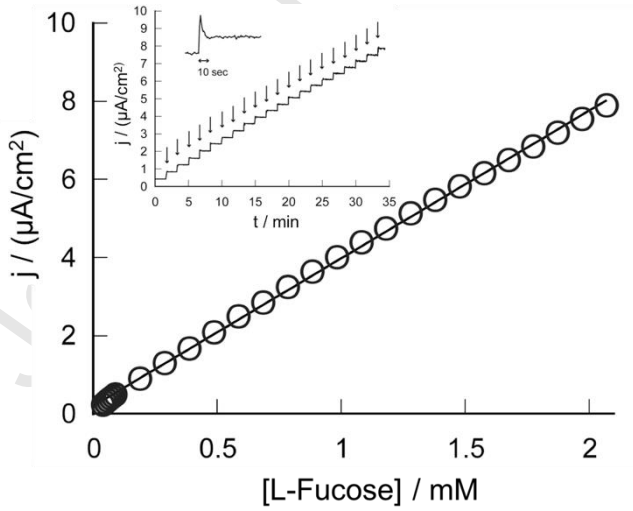


Fig. 7. Dependence of current response on L-fucose concentration at the *Cc*PDH immortalized carbon nanoparticle modified electrode in 0.1 M phosphate buffer (pH 8.0) at the potential of +0.1 V vs. Ag|AgCl, 3 M NaCl. The electrode stirring rate: 500 rpm. Liner relationship between the currents and the range of L-fucose concentrations (0.04 to 2.1 mM). The sensor sensitivity was 4.54 mA cm^{-2} mM^{-1} (r^2 = 0.9996). The inset is the actual response curve of sequential injections (arrows).

In practical use, direct detection of L-fucose in urine may be influenced by contaminating substances with electrochemical activity such as uric acid, dopamine and ascorbic acid. Depending on the working potential, these compounds may directly react with the electrode, causing high noise, and in this case, accurate sensing of L-fucose cannot be performed. At an operating potential of 0 V, the effects of uric acid and dopamine were negligible; however, the effects of ascorbic acid were profound (Fig. 8).

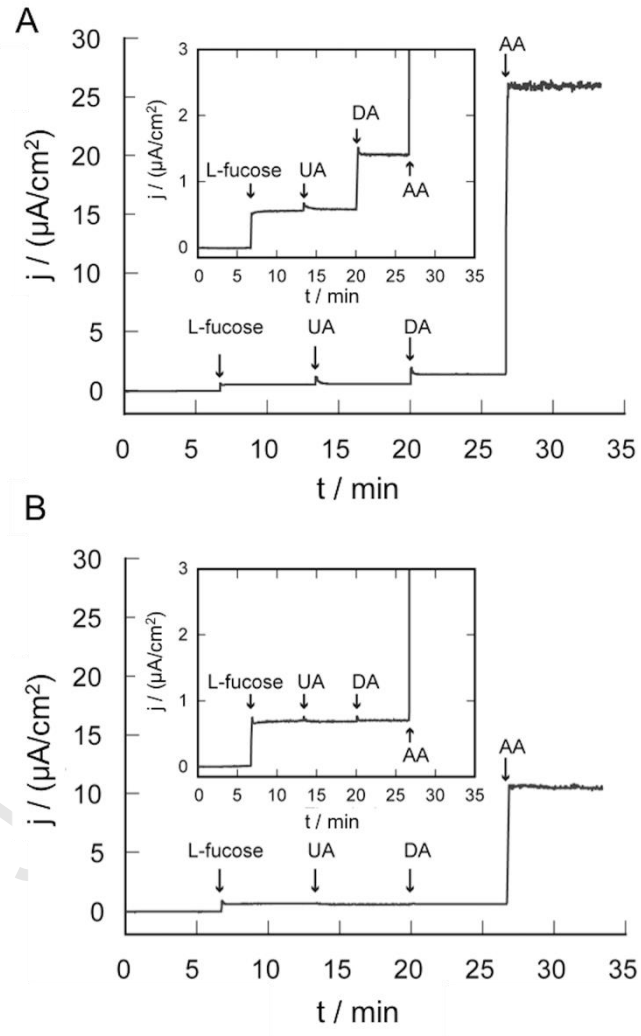
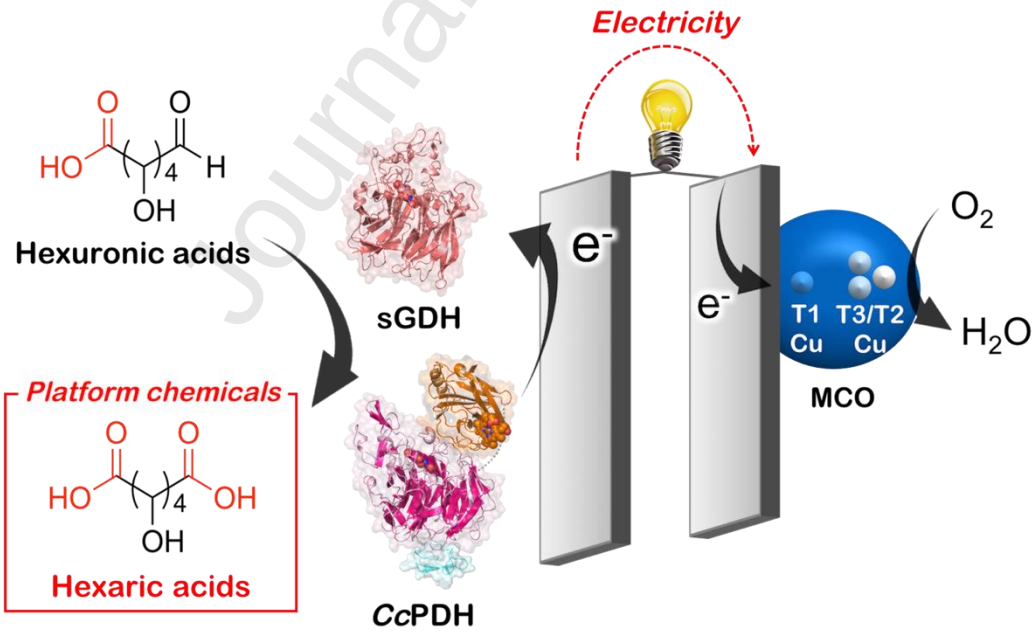


Fig. 8. Amperometric responses of *Cc*PDH immortalized carbon nanoparticle modified electrode to addition of 0.1 mM L-fucose, 1.9 mM uric acid (UA), 4.8 mM dopamine (DA), and 0.085 mM ascorbic acid (AA) in 100 mM phosphate buffer (pH 8.0) at the potential of (A) +0.1 V and (B) 0 V vs. Ag|AgCl, 3 M NaCl. The electrode stirring rate: 500 rpm. The insets show the responses in narrow current ranges.

To eliminate the influence of interfering substances, the following methods can be considered. (1) Interfering substances are removed by pretreatment of urine samples. (2) The sensor part is modified so that contaminating substances do not react directly with the electrode. (3) The measurement is performed at an operating potential that is lower than the oxidation potential of the interfering substances. Our group is working on a fundamental solution involving development of a sensor that operates at a potential that is not affected by ascorbic acid and the results will be reported.

5. Simultaneous production of hexaric acid and electric power using an enzymatic biofuel cell

When chemical production involves oxidation reactions, the extracted electrons are usually discarded to an electron acceptor such as dioxygen. However, the electrode can also be used as an electron acceptor for enzyme-catalyzed oxidation reactions. If an oxidative biomass-derived chemical conversion reaction catalyzed by an enzyme on the anode can be combined, for example, with an oxygen reduction reaction at the cathode, electrical energy can be generated. Therefore, in our laboratory, we aim for the simultaneous production of chemical products and electrical energy from biomass using a biofuel cell that uses enzymes as electrode catalysts (Scheme 1).



Scheme 1. Schematic description of an enzymatic biofuel cell that simultaneously produces a chemical compound and electricity. MCO means a multicopper oxidase.

Recently, it has been reported that PQQ-dependent soluble glucose dehydrogenase (sGDH; EC 1.1.99.35), which binds pyrroloquinoline quinone as a coenzyme, oxidizes D-galacturonic acid, D-glucuronic acid, and D-mannuronic acid at the C-1 position to form meso-galactaric acid, D-glucaric acid, and D-mannaric acid, respectively [45]. In particular, using an electrode modified with sGDH in a system with galacturonic acid as a substrate resulted in successful electrochemical oxidation of D-galacturonic acid [46]. As described above, *Cc*PDH is expected to oxidize L-guluronic acid as well because *Cc*PDH can oxidize the C-1 position of a pyranose in the reverse-chair conformation ($^1C_4$). In fact, the catalytic domain of *Cc*PDH was shown to catalyze the oxidation of the C-1 position of L-guluronic acid to form D-glucaric acid [45]. These enzymes may be suitable for use in a system for simultaneous production of chemical products and electrical energy. It will be possible to produce chemical products that are conventionally produced by applying energy while obtaining electrical energy. This approach may represent a highly efficient method of biomass conversion, which has never been described previously. The proposed simultaneous production principle can be applied to the production of compounds other than biomass as long as the production is carried out by a redox reaction. Enzymes capable of direct electron transfer with the electrode will become increasingly important.

6. Conclusions

This review describes the characterization of the newly discovered quinohemoprotein *Cc*PDH, the development of an amperometric sensor using *Cc*PDH as an electrocatalyst, and the research using *Cc*PDH as an anode catalyst for a biofuel cell. The cytochrome *b* domain enables to direct electron transfer between the catalytic domain and the electrode, as well as the heme domains of the quinohemoprotein alcohol dehydrogenases and the flavohemoprotein CDHs. The results show that *Cc*PDH exhibits DET based bioelectrocatalysis. A heterologous overexpression system of *Cc*PDH has been constructed by using *P. pastoris*, so that various kinds of enzyme variants will be conveniently generated. Therefore, this enzyme is very useful for bioelectronics devices. In addition, this type of cytochrome b domain could be used as an electron mediator from the catalytic site of various FAD or PQQ-dependent oxidoreductases to the electrode. If enzymes corresponding to the redox reactions of various chemical substances are identified, it is expected that they will lead to the development of sensing devices and electrochemical conversion systems for these chemical substances.

Another important research objective relates to the domain interaction mechanism when IET reaction occurs in *Cc*PDH. Evidence suggests that the IET

reaction depends the pH and the difference in redox potential between PQQ and heme. The structural analysis of the cytochrome *b* domain implies that the pH-dependent IET process may be caused by the positive charge around heme. As figured out in CDH so far, the mechanism of electrostatic interaction between the domains of *Cc*PDH is of interest for intramolecular electron transfer. To achieve a deeper and more detailed understanding of the electron transfer pathway, structural analysis of full-length *Cc*PDH (the conformational state for productive IET) is urgently needed, as well as the kinetics analysis of PQQ and heme *b* reduction rate in the pre-steady-state of *Cc*PDH and its variants. These analyses would be helpful for understanding of the intramolecular electron transfer of other multi-cofactor enzymes and also inter-protein electron transfer reaction.

We also focus our attention on an evolutionary aspect of eukaryotic PQQ-dependent dehydrogenases. How was the PQQ enzyme gene integrated into eukaryotes? Despite secretory enzymes, how is PQQ supplied to apo-enzyme in the environment? Does symbiosis exist with bacteria that can synthesize PQQ? It is expected that researches will be conducted to elucidate these questions.

Acknowledgements

This work was financially supported by a Grant-in-Aid for Young Scientific Research (B), JSPS KAKENHI Grant Number 17K17703 to KT.

References

[1] S.A. Salisbury, H.S. Forrest, W.B. Cruse, O. Kennard, A novel coenzyme from bacterial primary alcohol dehydrogenases, Nature, 280 (1979) 843-844.

[2] J. Westerling, J. Frank, J.A. Duine, The prosthetic group of methanol dehydrogenase from Hyphomicrobium X: electron spin resonance evidence for a quinone structure, Biochem Biophys Res Commun, 87 (1979) 719-724.

[3] A. Oubrie, Structure and mechanism of soluble glucose dehydrogenase and other PQQ-dependent enzymes, Biochim Biophys Acta, 1647 (2003) 143-151.

[4] C. Anthony, The quinoprotein dehydrogenases for methanol and glucose, Arch Biochem Biophys, 428 (2004) 2-9.

[5] H. Toyama, F.S. Mathews, O. Adachi, K. Matsushita, Quinohemoprotein alcohol dehydrogenases: structure, function, and physiology, Arch Biochem Biophys, 428 (2004) 10-21.

[6] R.L. Arechederra, S.D. Minteer, Complete Oxidation of Glycerol in an Enzymatic Biofuel Cell, Fuel Cells, 9 (2009) 63-69.

[7] S. Xu, S.D. Minteer, Enzymatic Biofuel Cell for Oxidation of Glucose to CO2, Acs Catal, 2 (2012) 91-94.

[8] K. Takeda, H. Matsumura, T. Ishida, M. Samejima, K. Igarashi, N. Nakamura, H. Ohno, The two-step electrochemical oxidation of alcohols using a novel recombinant PQQ alcohol dehydrogenase as a catalyst for a bioanode, Bioelectrochemistry, 94 (2013) 75-78.

[9] H. Matsumura, K. Umezawa, K. Takeda, N. Sugimoto, T. Ishida, M. Samejima, H. Ohno, M. Yoshida, K. Igarashi, N. Nakamura, Discovery of a eukaryotic pyrroloquinoline quinone-dependent oxidoreductase belonging to a new auxiliary activity family in the database of carbohydrate-active enzymes, PLoS One, 9 (2014) e104851.

[10] K. Takeda, H. Matsumura, T. Ishida, M. Samejima, H. Ohno, M. Yoshida, K. Igarashi, N. Nakamura, Characterization of a novel PQQ-dependent quinohemoprotein pyranose dehydrogenase from Coprinopsis cinerea classified into auxiliary activities family 12 in carbohydrate-active enzymes, PLoS One, 10 (2015) e0115722.

[11] T. Yakushi, K. Matsushita, Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology, Appl Microbiol Biotechnol, 86 (2010) 1257-1265.

[12] A. Ramanavicius, K. Habermuller, E. Csoregi, V. Laurinavicius, W. Schuhmann, Polypyrrole-entrapped quinohemoprotein alcohol dehydrogenase. Evidence for direct electron transfer via conducting-polymer chains, Anal Chem, 71 (1999) 3581-3586.

[13] W. Schuhmann, H. Zimmermann, K. Habermuller, V. Laurinavicius, Electron-transfer pathways between redox enzymes and electrode surfaces: reagentless biosensors based on

thiol-monolayer-bound and polypyrrole-entrapped enzymes, Faraday Discuss, (2000) 245-255; discussion 257-268.

[14] J. Tkac, J. Svitel, I. Vostiar, M. Navratil, P. Gemeiner, Membrane-bound dehydrogenases from Gluconobacter sp.: interfacial electrochemistry and direct bioelectrocatalysis, Bioelectrochemistry, 76 (2009) 53-62.

[15] S. Xu, S.D. Minteer, Investigating the Impact of Multi-Heme Pyrroloquinoline Quinone-Aldehyde Dehydrogenase Orientation on Direct Bioelectrocatalysis via Site Specific Enzyme Immobilization, Acs Catal, 3 (2013) 1756-1763.

[16] S. Ma, R. Ludwig, Direct Electron Transfer of Enzymes Facilitated by Cytochromes, ChemElectroChem, 6 (2019) 958-975.

[17] K. Takeda, T. Ishida, M. Yoshida, M. Samejima, H. Ohno, K. Igarashi, N. Nakamuraa#, The Crystal Structure of the Catalytic Domain and the Cytochrome b Domain in a Eukaryotic PQQ-Dependent Dehydrogenase, manuscript submitted.

[18] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch, Protein Identification and Analysis Tools on the ExPASy Server; (In) John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press 2005.

[19] A. Varnai, K. Umezawa, M. Yoshida, V.G.H. Eijsink, The pyrroloquinoline-quinone dependent pyranose dehydrogenase from Coprinopsis cinerea (CcPDH) drives lytic polysaccharide monooxygenase (LPMO) action, Appl Environ Microbiol, (2018).

[20] S. Igarashi, T. Hirokawa, K. Sode, Engineering PQQ glucose dehydrogenase with improved substrate specificity. Site-directed mutagenesis studies on the active center of PQQ glucose dehydrogenase, Biomol Eng, 21 (2004) 81-89.

[21] A.J. Olsthoorn, J.A. Duine, On the mechanism and specificity of soluble, quinoprotein glucose dehydrogenase in the oxidation of aldose sugars, Biochemistry, 37 (1998) 13854-13861.

[22] G.E. Cozier, R.A. Salleh, C. Anthony, Characterization of the membrane quinoprotein glucose dehydrogenase from Escherichia coli and characterization of a site-directed mutant in which histidine-262 has been changed to tyrosine, Biochem J, 340 (Pt 3) (1999) 639-647.

[23] J.D. Cohen, W. Bao, V. Renganathan, S.S. Subramaniam, T.M. Loehr, Resonance Raman spectroscopic studies of cellobiose dehydrogenase from Phanerochaete chrysosporium, Arch Biochem Biophys, 341 (1997) 321-328.

[24] F.A. Rotsaert, B.M. Hallberg, S. de Vries, P. Moenne-Loccoz, C. Divne, V. Renganathan, M.H. Gold, Biophysical and structural analysis of a novel heme B iron ligation in the flavocytochrome cellobiose dehydrogenase, J Biol Chem, 278 (2003) 33224-33231.

[25] K. Takeda, K. Umezawa, A. Varnai, V.G. Eijsink, K. Igarashi, M. Yoshida, N. Nakamura, Fungal PQQ-dependent dehydrogenases and their potential in biocatalysis, Curr Opin

Chem Biol, 49 (2018) 113-121.
[26] K. Umezawa, K. Takeda, T. Ishida, N. Sunagawa, A. Makabe, K. Isobe, K. Koba, H. Ohno, M. Samejima, N. Nakamura, K. Igarashi, M. Yoshida, A Novel Pyrroloquinoline Quinone-Dependent 2-Keto-D-Glucose Dehydrogenase from Pseudomonas aureofaciens, J Bacteriol, 197 (2015) 1322-1329.
[27] K. Takeda, H. Matsumura, T. Ishida, M. Yoshida, K. Igarashi, M. Samejima, H. Ohno, N. Nakamura, pH-dependent electron transfer reaction and direct bioelectrocatalysis of the quinohemoprotein pyranose dehydrogenase, Biochem Biophys Res Commun, 477 (2016) 369-373.
[28] L. Stoica, R. Ludwig, D. Haltrich, L. Gorton, Third-generation biosensor for lactose based on newly discovered cellobiose dehydrogenase, Anal Chem, 78 (2006) 393-398.
[29] H. Matsumura, R. Ortiz, R. Ludwig, K. Igarashi, M. Samejima, L. Gorton, Direct electrochemistry of Phanerochaete chrysosporium cellobiose dehydrogenase covalently attached onto gold nanoparticle modified solid gold electrodes, Langmuir, 28 (2012) 10925-10933.
[30] R. Ludwig, W. Harreither, F. Tasca, L. Gorton, Cellobiose dehydrogenase: a versatile catalyst for electrochemical applications, Chemphyschem, 11 (2010) 2674-2697.
[31] R. Ludwig, R. Ortiz, C. Schulz, W. Harreither, C. Sygmund, L. Gorton, Cellobiose dehydrogenase modified electrodes: advances by materials science and biochemical engineering, Anal Bioanal Chem, 405 (2013) 3637-3658.
[32] O. Berteau, B. Mulloy, Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide, Glycobiology, 13 (2003) 29r-40r.
[33] M.T. Ale, J.D. Mikkelsen, A.S. Meyer, Important determinants for fucoidan bioactivity: a critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds, Mar Drugs, 9 (2011) 2106-2130.
[34] E. Deniaud-Bouet, K. Hardouin, P. Potin, B. Kloareg, C. Herve, A review about brown algal cell walls and fucose-containing sulfated polysaccharides: Cell wall context, biomedical properties and key research challenges, Carbohydr Polym, 175 (2017) 395-408.
[35] H.M. Flowers, Chemistry and biochemistry of D- and L-fucose, Adv Carbohydr Chem Biochem, 39 (1981) 279-345.
[36] M.A. Lehrman, S.V. Pizzo, M.J. Imber, R.L. Hill, The binding of fucose-containing glycoproteins by hepatic lectins. Re-examination of the clearance from blood and the binding to membrane receptors and pure lectins, J Biol Chem, 261 (1986) 7412-7418.
[37] B. Condaminet, J. Peguet-Navarro, P.D. Stahl, C. Dalbiez-Gauthier, D. Schmitt, O. Berthier-Vergnes, Human epidermal Langerhans cells express the mannose-fucose binding

receptor, Eur J Immunol, 28 (1998) 3541-3551.
[38] T. Tatsumura, H. Sato, A. Mori, Y. Komori, K. Yamamoto, G. Fukatani, S. Kuno, Clinical significance of fucose level in glycoprotein fraction of serum in patients with malignant tumors, Cancer Res, 37 (1977) 4101-4103.
[39] M. Endo, H. Matsue, S. Sato, M. Majima, N. Hiyama, Enzymic determination of urinary free L-fucose, Clin Chim Acta, 103 (1980) 269-275.
[40] Y. Deugnier, V. David, P. Brissot, P. Mabo, D. Delamaire, M. Messner, M. Bourel, J.Y. Legall, Serum alpha-L-fucosidase: a new marker for the diagnosis of primary hepatic carcinoma?, Hepatology, 4 (1984) 889-892.
[41] G.A. Turner, A.W. Skillen, P. Buamah, D. Guthrie, J. Welsh, J. Harrison, A. Kowalski, Relation between raised concentrations of fucose, sialic acid, and acute phase proteins in serum from patients with cancer: choosing suitable serum glycoprotein markers, J Clin Pathol, 38 (1985) 588-592.
[42] P.S. Patel, S.G. Adhvaryu, D.B. Balar, B.J. Parikh, P.M. Shah, Clinical application of serum levels of sialic acid, fucose and seromucoid fraction as tumour markers in human leukemias, Anticancer Res, 14 (1994) 747-751.
[43] T. Sakai, K. Yamamoto, H. Yokota, K. Hakozaki-Usui, F. Hino, I. Kato, Rapid, simple enzymatic assay of free L-fucose in serum and urine, and its use as a marker for cancer, cirrhosis, and gastric ulcers, Clin Chem, 36 (1990) 474-476.
[44] C.G. Tsiafoulis, M.I. Prodromidis, M.I. Karayannis, Development of an amperometric biosensing method for the determination of L-fucose in pretreated urine, Biosens Bioelectron, 20 (2004) 620-627.
[45] R. Sakuta, K. Takeda, K. Igarashi, H. Ohno, N. Nakamura, Enzymes Suitable for Biorefinery to Coproduce Hexaric Acids and Electricity from Hexuronic Acids Derived from Biomass, Energy Technol-Ger, 6 (2018) 273-279.
[46] R. Sakuta, KoutaTakeda, K. Igarashi, H. Ohno, N. Nakamura, Pyrroloquinoline quinone-dependent glucose dehydrogenase anode: D-Galacturonic acid oxidation and galactaric acid production, Journal of Molecular Catalysis B: Enzymatic, 133 (2016) S76-S79.

Graphical abstract:

Highlights

- The electron transfer reaction of *Cc*PDH is summarized.
- A biosensor of L-fucose, a cancer marker, was prepared using *Cc*PDH.

- *Cc*PDH is also useful as an anode catalyst for DET-type biofuel cells.