Talanta 80 (2009) 19–23

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Spectrofluorimetric method for measuring the activity of the enzyme α-L-fucosidase using the ion associate of 2-chloro-4-nitro phenol–rhodamine-B

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ARTICLE INFO

Article history:
Received 10 February 2009
Received in revised form 11 April 2009
Accepted 13 April 2009
Available online 11 June 2009

Keywords:
α-L-Fucosidase enzyme
Rhodamine-B
2-Chloro-4-nitrophenol
Associate

ABSTRACT

A low cost and accurate method for the detection and analytical determination of the activity of the enzyme α-L-fucosidase (AFU) was developed. The method was based upon measuring the fluorescence intensity of the complex ion associate of the ion associate of rhodamine-B and the compound 2-chloro-4-nitrophenol (RB^+ CNP^-) at 580 nm in phosphate buffer (pH 5) against the reagent blank. The influence of the different parameters, e.g. pH, incubation time, temperature, 2-chloro-4-nitrophenol concentration, foreign ions and surfactants that control the fluorescence intensity of the produced ion associate was critically investigated. The correlation between the fluorescence activity of the enzyme AFU by the developed procedures and the standard method was positive and highly significant in patients and controls ($r^2 = 0.99$, $p < 0.001$). The developed method is simple and proceeds without practical artifacts compared to the standard method.

1. Introduction

Liver cirrhosis is a pre-cancer condition that in many cases can develop into Hepatocellular carcinoma (HCC). Therefore, cirrhotic patients with cirrhosis are usually screened for HCC during their follow-up procedure [1–6]. Tumor markers represent good potential screening tools for the early diagnosis of tumors [7–10]. The primary tumor marker for HCC is a single polypeptide chain glycoprotein namely α-fetoprotein (AFP) [11,12]. The sensitivity and specificity of the AFP as a common tumor marker to detect HCC in all patient samples are insufficient [13,14]. Moreover, it is not secreted in all cases of HCC and may be normal in 40% of patients with early HCC [10,15].

The enzyme α-L-Fucosidase (AFU) can hydrolyze methyl α-L-fucoside and fucosidic linkages of fucoidan and blood-group substances [16]. The AFU enzyme compose of two-enzyme components namely: i, fucosidase which acts on 4-nitrophenyl α-L-fucoside as well as fucosidic linkages of porcine sub-maxillary mucin at optimal activity at pH 2 and ii, the fucoidanase showed highest activity around pH 5 and acts on the synthetic substrate not on the mucin [17]. The enzyme fucoidanase showed hydrolytic activity towards fucoidan, and not towards 4-nitro phenyl α-L-fucoside and blood group of livers of related species [18]. Clinical studies have demonstrated that, the activity of AFU enzyme represent an excellent test for diagnosis of HCC [19,20]. The AFU enzyme was used for the diagnosis of fucosidosis recognized in born disorder of metabolism and increases the sensitivity of detection to 95.5% in patients with HCC [21,22].

Different spectrometric methods have been reported for the determination of the activity of the enzyme AFU in serum [23–25]. These methods are limited by their long incubation time (30–60 min) for the sample and reagent blanks. Moreover, the poor affinity of the enzyme AFU towards the substrate 4-NPF as a colorimetric reagent at pH 4.8, added another disadvantage to these methods. However, the method of Jun and Hua [23] involving the synthesis of 2-chloro-4-nitrophenyl-α-L-fucopyranoside (CNPF) as a substrate has a rapid hydrolytic rate than 4-NPF method [23,24]. The activity of AFU was also determined by spectrofluorescence methods [26,27], affinity chromatography [17], column chromatography packed with Bacillus cereus [28] and disc gel electrophoresis technique [17,29]. Most of these methods are unselective, require careful experimental conditions, considerably time consuming and not compatible to detect the activity of AFU at the early stage of diseases. Thus, the present manuscript is focused on the use of the fluorescent ion associate of 2-chloro-4-nitro phenol–rhodamine-B (RB^+ CNP^-) for measuring the activity of the AFU enzyme and overcoming the difficulties caused by the previous methods [23–27]. The developed procedures will be able to control the quality of

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0039-9140/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.talanta.2009.04.070

the final marketed product in both qualitative and quantitative approaches to assure the identity of the disease. The method will be useful in medical applications to detect the different diseases at the very early stage of diseases.

2. Experimental

2.1. Apparatus

A Shimadzu RF5301 PC spectrofluorometer (290–750 nm) was used for recording the excitation; emission spectra and measuring the fluorescence intensity of the reagents and the produced complex ion associate. A two matched cuvettes (1.0 cm) and an Orion pH meter VWR scientific model (8000) were used for measuring the fluorescence intensity of the ion associate $RB^+ CN^-$ and other fluorescent species and pH measurements, respectively.

2.2. Reagents and materials and methods

Analytical reagent grade (A.R.) reagents and chemicals (99.9% purity) were used without further purification. Ascorbic acid, bilirubin, hemoglobin, glucose and uric acid, albumin, total protein, total cholesterol and triglyceride were purchased from Fluka (Fluka, AG, Buchs, Switzerland). The substrate 2-chloro-4-nitrophenyl-α-L-fucopyranoside (CNPF) and the enzyme α-L-fucosidase (AFU, EC 3.2.1.51) were laboratory reagent grade from Sigma–Aldrich, Poole, UK. The control enzyme was prepared in bovine serum base and provided in lyophilized powder. The enzyme AFU was also obtained from bovine kidney and the reagents 2-chloro-4-nitro phenol (CNP) and rhodamine-B (RB) were purchased from Sigma Co. Phosphate buffers of various pH (pH 2.5–7) were prepared from phosphoric acid and/or sodium phosphate.

2.3. Preparation of the complex ion associate $RB^+ CNP^-$

In measuring flask (100.0 mL), an accurate volume (10 mL) of the reagent 2-chloro-4-nitrophenol (5×10^{-4} mol L^{-1}) was allowed to react with 20.0 mL of rhodamine-B (5×10^{-4} mol L^{-1}). The solution was then completed to the mark with phosphate buffer of pH 5.0 and the excitation and emission spectra of the reagents 2-chloro-4-nitrophenol and rhodamine-B and the produced complex ion associate ($RB^+ CNP^-$) were then recorded.

2.4. Analytical application

An appropriate amount (450 μL) of the reagent 2-chloro-4-nitro-phenol fucopy-ranoside was immediately mixed with 50 μL of plasma/serum samples of HCC patients (26 persons) and healthy control (7 persons)) in incubator at 37 °C for 6 min. To the resultant solutions an accurate volume (300 μL) of rhodamine-B (5×10^{-4} mol L^{-1}) was added and swirled to mix the contents. Each solution was placed in an incubator at 37 °C for 5 min. The volume was completed to 3.0 mL with phosphate buffer of pH 5 and the emission intensity at 580 nm was then measured against the reagent blank. The main characteristics of the patients (male/female) and control groups are given in Table 1.

3. Result and discussion

3.1. Excitation and emission spectra

Previous studies [26] have shown that, the enzyme AFU reacts with the substrate 2-chloro-4-nitrophenol or 4-nitrophenol at pH 4–5 producing yellow colored product. This reaction has been used for measuring the activity of the enzyme AFU via the absorbance of the produced yellow colored species at pH 5.0 and 37 °C [24]. However, this, method suffered from the interference caused by the yellow color of the serum of patients and of the reagent 2-chloro-4-nitrophenol (or 4-nitro phenol). Another disadvantage was also noticed in many cases of patients of HCC that have high bilirubin [22,23]. Such methods suffered from the lake of sensitivity and accuracy due to the significant interference of the yellow color of the serum. Thus, attempts were made to overcome these disadvantages by testing the interaction of the compound 4-nitro phenol or 2-chloro-4-nitro phenol with rhodamine-B dye in aqueous medium.

In phosphate buffer (pH 5), preliminary investigations showed that, on mixing the reagent CNP with the ion pairing reagent RB and shaking, a green colored complex ion associate was developed and is most likely proceeds according to the following equation:

$$\text{2-Chloro-4-nitrophenol} + \text{Rhodamine-B} \rightleftharpoons [\text{Rhodamine-B}]^+ \text{ [2-Chloro-4-NP]-Ion associate} \quad (1)$$

After equilibrium, the excitation and emission spectra of the produced complex ion associate of rhodamine-2-chloro-4-nitrophenol or rhodamine-4-nitro phenol ($RB^+ CNP^-$) in the aqueous phase at pH 5 were recorded. The excitation and emission spectra (Fig. 1) of the complex associate $RB^+ CNP^-$ showed the characteristic excitation wavelength at 455 nm and the characteristic emission wavelength at 580 nm. The fluorescence intensity of the rhodamine-B in the emission spectrum at 580 nm decreased on adding CNP confirming the associate formation. On the other hand, rhodamine-B showed well defined emission spectrum (Fig. 2) close to the emission spectrum of the complex ion associate $RB^+ CNP^-$ (Fig. 1) while, compound 2-chloro-4-nitrophenol or 4-nitro phenol showed only excitation spectrum and no emission spectrum at 580 nm. Thus, in the subsequent work, the activity of the enzyme AFU was determined through measuring the fluorescence intensity of the ion associate $RB^+ CNP^-$ at 580 nm against a reagent blank at the optimum experimental conditions.

3.2. Analytical parameters

The influence of the hydrogen ion concentration on the fluorescence intensity of the complex ion associate $RB^+ CNP^-$ was investigated. The emission spectrum of the solution of 2-chloro-4-NP (5×10^{-5} mol L^{-1}) in the presence of rhodamine-B (5×10^{-5} mol L^{-1}) in a wide range of pH 2–8 employing dilute HCl and/or dilute sodium hydroxide was measured. The results shown in Fig. 3 revealed maximum change in the emission intensity in the pH range pH 2.5–6.0. At pH less than pH 2.5, the emission intensity of the produced complex associate $RB^+ CNP^-$ at 580 nm

Table 1
Main characteristics of the patients and control groups.

Status	Number of patients	Males/females		Age (year)	
		No.	%	Median	Range
HCC	20	15/5	75/25	51	24–75
Cirrhosis of chronic hepatitis C and B	4	2/2	50/50	48	37–69
Other neoplasms (gallbladder cancer, colon cancer and others)	2	2/0	100/0	42	28–56
Healthy adults	7	4/3	60/45	36	19–58