Use of a New Enzyme Extraction System to Improve the Sensitivity of SOS/*umu* Test and Application to Environmental Samples

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Abstract The purpose of this study was to find a better enzyme extraction reagent for the SOS/*umu* test to replace the conventional one (the combination of sodium dodecyl sulfate (SDS) and Z-buffer), which has the disadvantage of denaturing β -galactosidase leading to decreased measurement sensitivity. By adopting a microplate system, the performance of the *umu* test using BugBuster Master Mix, a commercially available enzyme extraction reagent, was compared with that using the conventional reagent for detecting the genotoxicity of known mutagens as well as environmental samples. BugBuster Master Mix was found to increase the detection sensitivities of the selected genotoxins and environmental water samples, due to the fact that it doesn't denature β -galactosidase. The result of this study showed that BugBuster Master Mix could be a better enzyme extraction reagent for *umu* test.

Keywords SOS/*umu* test · β -Galactosidase activity · BugBuster Master Mix · Sodium dodecyl sulfate treatment · Genotoxicity

With increasing numbers of chemical substances being discharged into the environment, evaluation and monitoring of the impacts of these chemicals to the water environment and human health is becoming more and more important (Ohe et al. 2004). Rapid and sensitive bioassay systems have been developed for detecting environmental genotoxins as a whole based on the ability of these substances to cause DNA damage (Monarca et al. 2004).

Among different bioassays for the detection of environmental mutagens, the SOS/*umu* test, which monitors the expression of one of the SOS genes (*umuC* gene) by measuring β -galactosidase activity (Oda et al. 1985), has attracted vast attention because of its simplicity, short test time and low requirement for disinfection (Hamer et al. 2000). By employing a single *Salmonella typhimurium* tester strain (*S. typhimurium* TA1535/pSK1002), this bioassay system allows the detection of SOS mutagenesis after treatment with DNA-damaging agents (Michel 2005). As the standardized method of ISO (ISO/CD 13829) (ISO 2000) and DIN (DIN 38415-3) (DIN 1996), the SOS/*umu* test has been extensively employed to detect genotoxicity in different media including airborne particles (Oda et al. 2004), tap water (Shen et al. 2003), reclaimed water (Wu et al. 2010) and industrial wastewater (Dizer et al. 2002).

Enzyme extraction is one of the important steps in the SOS/*umu* test to detect the induced activity of β -galactosidase. In comparison with toluene, the frequently used protein extraction detergent, sodium dodecyl sulfate (SDS), demonstrated its advantage in shortening test time and increasing induced β -galactosidase activity (Whong et al. 1986), and the combination of SDS and Z-buffer (Miller 1972) has been widely used as the enzyme extraction system for the SOS/*umu* test. However, SDS treatment has been reported to denature β -galactosidase (Demeo et al. 1988; El Mzibri et al. 1996; Whong et al. 1986) and may result in poor protein recovery (De Mey et al. 2008), which can decrease the measurement sensitivity of β -galactosidase activity. As an effective protein extraction detergent, BugBuster Master Mix (B. M. Mix) is a specially formulated reagent combining BugBuster Protein Extraction Reagent with Benzonase[®] Nuclease and rLysozyme[™] Solution (Novagen[®] and Calbiochem[®] 2004). The use of non-ionic detergents in BugBuster Protein Extraction Reagent permits cell wall perforation without denaturing

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target proteins, while the ability to hydrolyze *N*-acetylmuramide linkages in cell walls by rLysozymeTM Solution may enhance the extraction efficiency of soluble proteins, and the ability to degrade all forms of DNA and RNA by benzonase makes the resulting extract less viscous and more clarified, all of which are desirable features in a protein extraction reagent (Grabski et al. 1999; Novagen[®] and Calbiochem[®] 2006). Recently, Grage et al. (2011) has applied B. M. Mix to the assay of β -galactosidase to assess the functionality of purified recombinant protein. However, this reagent never was used for enzyme extraction in the *umu* test instead of the conventional system (the combination of SDS and Z-buffer).

In this paper, the applicability of B. M. Mix for the SOS/*umu* test was evaluated by comparing its performance with that of the SDS-Z-buffer system in detecting the genotoxicity of some pure chemicals as well as a variety of environmental water samples. The results suggested that B. M. Mix, as an effective enzyme extraction reagent, could increase the sensitivity of the SOS/*umu* test.

Materials and Methods

The test chemicals were obtained from the following sources: 4-nitroquinoline 1-oxide (4-NQO), methyl methanesulfonate (MMS), 5-fluorouracil (5-FU), streptozotocin, 2-aminoanthracene (2-AA) and benzo[*a*]pyrene (B[*a*]P) from Sigma–Aldrich (St. Louis, MO, USA); 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The enzyme substrate chlorophenol red- β -D-galactopyranoside (CPRG) was purchased from Sigma–Aldrich (St. Louis, MO, USA). BugBuster Master Mix (B. M. Mix in short) protein extraction reagent (Novagen[®]) was obtained from EMD Chemicals, Inc. (San Diego, CA, USA).

Twenty-four-h representative wastewater and reclaimed water samples were collected from different positions of several municipal wastewater treatment and reclamation plants in Beijing and Tianjin, China. At the same time, three drinking water samples (D9–D11) were collected from the finished water tanks of different cities, and eight samples (D1–D8) were collected from a pilot system treating source water from the Huangpu River in Shanghai, China, with a combined process (combination of coagulation-sedimentation-sand filtration and ozonation-biological activated carbon filtration). For chlorinated water samples, sodium thiosulfate (20 mg/1,000 mL) was added to quench chlorine residue. The samples were stored with ice during transportation, delivered to the laboratory as soon as possible, and then filtered through glass fiber filters (0.7 μ m) to eliminate suspended solids.

The filtered water samples were concentrated and prepared as described by Ma et al. (2012) with some modifications. Briefly, Oasis HLB solid extraction cartridges (6 cc 500 mg, Waters, USA) were conditioned before use with 10 mL of dichloromethane, 10 mL of acetone, 10 mL of methanol and 10 mL of distilled water in succession. 2.5 L drinking water, 1 L reclaimed water or 0.075 L wastewater sample was loaded onto one cartridge, respectively, then dried under a gentle nitrogen flow and eluted with 15 mL of acetone. The eluate was dried using nitrogen gas and the dried residues were reconstituted with 25 μ L dimethylsulfoxide (DMSO). The extracts were stored in the dark at -20°C before the *umu* test.

The modified microplate method developed by Oda et al. (2004) was used for the *umu* test. 2 μ L of water sample extracts or chemicals was added to the exponentially growing tester strain culture (98 μ L), and the mixture was incubated at 37°C for 2 h. Subsequently, in the step of detecting β -galactosidase activity, 10 μ L of the culture was transferred to a microplate with either the conventional extraction system (90 μ L Z-buffer and 50 μ L of 0.1 % SDS) (Oda et al. 2004) or B. M. Mix (50 μ L). The enzymatic reaction was started by the addition of 10 μ L of 4 mg/mL CPRG, and incubated for 20 min at 37°C , and

Table 1 Effects of the concentration of SDS and adding volume of B. M. Mix on induction of *umuC* gene expression by 4-NQO

4-NQO ($\mu\text{g/mL}$)	0		0.625		1.25	
	RGA $\pm$ SD	IR ^a	RGA $\pm$ SD	IR	RGA $\pm$ SD	IR
0.1 % SDS	0.79 $\pm$ 0.02	1.00	4.91 $\pm$ 0.31	6.23	12.10 $\pm$ 1.15	15.34
1 % SDS	0.63 $\pm$ 0.02	1.00	4.67 $\pm$ 0.10	7.39	9.65 $\pm$ 1.64	15.26
5 % SDS	0.39 $\pm$ 0.01	1.00	3.11 $\pm$ 0.18	7.93	5.50 $\pm$ 0.55	13.99
10 μL B. M. Mix	0.24 $\pm$ 0.01	1.00	1.45 $\pm$ 0.03	6.11	2.20 $\pm$ 0.27	9.25
50 μL B. M. Mix	0.61 $\pm$ 0.02	1.00	6.92 $\pm$ 0.46	11.33	10.28 $\pm$ 1.19	16.82
90 μL B. M. Mix	0.64 $\pm$ 0.04	1.00	7.02 $\pm$ 0.49	10.98	10.29 $\pm$ 0.85	16.10

^a IR The induction ratio (IR) was defined as the mean value of RGAs (adjusted A_{570}/A_{595}) at the tested doses divided by their values in the control

Fig. 1 Effects of time of B. M. Mix and SDS treatment on induction of *umuC* gene expression by 4-NQO. (Tester cells were treated with 0.625 $\mu\text{g}/\text{mL}$ of 4-NQO and in order to check the stability of β -galactosidase in SDS + Z-buffer lytic solution and B. M. Mix during the lytic period, CPRG was added to the cell-reaction mixture after various periods of lysis, and the β -galactosidase activity was assayed. Bar in figure represents standard deviation)

was stopped by adding 1 M Na_2CO_3 (100 μL). Absorbance at 570 nm (A_{570}) was read on the microplate reader to estimate the relative β -galactosidase activity (RGA). In this assay, DMSO was employed as a solvent control and a twofold increase in RGA above the control levels was estimated as positive. The results represented means of triplicate determinations. The genotoxicity of water sample was standardized to an equivalent 4-NQO or 2-AA concentration (4-NQO EQ or 2-AA EQ) (Wu et al. 2010).

Results and Discussion

In the enzyme assay step of the *umu* test, chemical cell disruption using proper detergents is vital for releasing

β -galactosidase from bacterial cells (Quillardet and Hofnung 1985; Whong et al. 1986), and in the modified *umu*-microplate test system of Oda et al. (2004) a combination of 90 μL Z-buffer and 50 μL 0.1 % SDS is used to lyse cells. Table 1 shows the β -galactosidase activities and induction ratio (IR) values under different enzyme extraction conditions with 4-NQO (0.625 or 1.25 $\mu\text{g}/\text{mL}$) as the positive marker. The detected β -galactosidase activity decreased with the increase of SDS concentration, showing that the use of SDS could lead to denaturation of β -galactosidase. When B. M. Mix was used, on the other hand, the β -galactosidase activity increased with the increase of B. M. Mix volume from 10 to 50 μL . However, further increase of B. M. Mix volume to 90 μL did not lead to the increase of β -galactosidase activity. Thus, the optimum application volume of B. M. Mix was determined to be 50 μL . The B. M. Mix system exhibited significantly higher IR values (7.02) than 4.91 of the conventional one employing SDS, particularly for the low exposure concentration of 4-NQO (0.625 $\mu\text{g}/\text{mL}$).

The effects of both enzyme extraction agents on the stability of β -galactosidase were compared (Fig. 1). For the SDS treatment system, the β -galactosidase activity was relatively stable for the first 10 min, but then decreased gradually, ending up with a decrease of 47 % after 40-min treatment, which was in accordance with previous reports (Demeo et al. 1988; El Mzibri et al. 1996; Whong et al. 1986). For the B. M. Mix treatment system, on the other hand, the β -galactosidase activity was almost constant over a period of 40 min. It has been reported that the detergent mixture in B. M. Mix was capable of cell wall perforation without denaturing proteins (Novagen® and Calbiochem® 2004). Therefore, B. M. Mix was favorable in terms of maintaining the stability of β -galactosidase, which was important for ensuring the measurement accuracy of the *umu* test.

Fig. 2 Effects of B. M. Mix and SDS treatment on induction of *umuC* gene expression by various chemicals

Table 2 The effects of B. M. Mix and SDS treatment on induction of *umuC* gene expression by water samples

Samples		B. M. Mix				0.1 %SDS + Z-Buffer			
		Positive/ negative (mL) ^a	SOSIA ^b (IR/mL)	4-NQO or 2-AA EQ (μg/L)	Carcinogenic risk ^c ($\times 10^{-6}$)	Positive/ negative (mL) ^a	SOSIA ^b (IR/mL)	4-NQO or 2-AA EQ (μg/L)	Carcinogenic risk ^c ($\times 10^{-6}$)
D	D1	–(200)	–	–	–	–(200)	–	–	–
	D2	–(200)	–	–	–	–(200)	–	–	–
	D3	–(200)	0.0013	0.0053	0.0554	–(200)	–	–	–
	D4	–(200)	0.0012	0.0046	0.0486	–(200)	–	–	–
	D5	–(200)	0.0017	0.0062	0.0654	–(200)	–	–	–
	D6	–(200)	0.0010	0.0038	0.0399	–(200)	–	–	–
	D7	–(200)	0.0012	0.0044	0.0468	–(200)	–	–	–
	D8	–(200)	0.0008	0.0028	0.0290	–(200)	–	–	–
	D9	+(100)	0.0110	0.0627	0.6606	–(200)	0.0019	0.0111	0.1172
	D10	+(25)	0.0488	0.2774	2.9245	–(200)	0.0065	0.0375	0.3949
	D11	–(125)	0.0035	0.0199	0.2096	–(125)	0.0024	0.0129	0.1355
R	R1	+(20)	0.0858	0.5645		+(40)	0.0294	0.3698	
	R2	–(80)	0.0035	0.0230		–(80)	–	–	
	R3	–(80)	0.0033	0.0216		–(80)	–	–	
	R1 (+S9)	+(20)	0.0910	7.3344		+(40)	0.0888	7.4363	
	R2 (+S9)	–(80)	0.0027	0.2176		–(80)	–	–	
	R3 (+S9)	–(80)	–	–		–(80)	–	–	
	R4	–(40)	0.0287	0.2148		–(40)	0.0053	0.1292	
	R5	+(20)	0.0802	0.6003		–(40)	0.0117	0.2868	
	R6	+(40)	0.0264	0.1437		+(80)	0.0116	0.1420	
	R7	–(80)	0.0157	0.0853		–(80)	–	–	
	R8	+(40)	0.0252	0.1372		–(80)	0.0058	0.0703	
	R9	+(80)	0.0218	0.1191		–(80)	0.0015	0.0181	
	R10	+(80)	0.0147	0.0801		–(80)	0.0027	0.0327	
	R11	–(80)	–	–		–(80)	–	–	
	R12	+(40)	0.0296	0.1616		–(80)	0.0033	0.0403	
W	R13	+(40)	0.0366	0.1993		–(80)	0.0064	0.0775	
	R14	+(20)	0.0782	0.4265		–(80)	0.0123	0.1499	
	W1	+(0.75)	2.0120	15.0553		+(1.5)	0.5803	14.1977	
	W1(+S9)	+(1.5)	1.5642	23.6175		+(1.5)	0.8843	17.0094	
	W2	–(2)	0.5271	3.8626		–(2)	0.1854	3.3283	

D drinking water, R reclaimed water, W wastewater

^a Positive/negative: “+”—positive; “–”—negative; the value in bracket represents the first volume (mL) detected showing positive response or the maximum volume tested with negative result

^b SOSIA: the SOS-inducing activity (SOSIA) was defined as the slope of the linear portion of the dose–response curve and was calculated by regression analysis

^c Carcinogenic risk: the carcinogenic risk of detected samples was calculated according to eq: carcinogenic risk ($\times 10^{-6}$) = 4-NQO EQ of the sample $\times 0.001 \times 0.369 \times 2 \times 10^6 / 70$ (Zhu et al. 2008)

The performance of the two extraction reagents in detecting *umuC* gene expressions induced by 10 chemicals in the presence and absence of S9 mix was compared as shown in Fig. 2. All the chemicals exhibited an increase of RGA in a dose-dependent fashion. In comparison with the SDS treatment, the use of B. M. Mix

generated stronger genotoxic responses for 4-NQO, MNNG, MMS, 5-FU, and streptozotocin without S9 mix, and 2-AA, B[a]P, IQ, Trp-P-1, and PhIP with S9 mix. Positive results were obtained at much lower exposure concentrations for compounds like 4-NQO, MNNG, MMS, 5-FU, and streptozotocin (Fig. 2).

Table 3 Genotoxic potencies of reclaimed water samples in the presence and absence of S9 mix

Samples	−S9			+S9		
	Positive/negative ^a	SOSIA (IR/mL)	4-NQO EQ (μg/L)	Positive/negative ^a	SOSIA (IR/mL)	2-AA EQ (μg/L)
G influent	+(10)	0.1510	0.7334	+(10)	0.1373	10.1704
G effluent	+(10)	0.1259	0.6115	+(20)	0.1113	8.2444
J influent	+(10)	0.1388	0.6741	+(10)	0.1434	10.6222
J effluent	+(80)	0.0195	0.0947	−(80)	0.0099	0.7333
Q influent	+(40)	0.0392	0.1904	+(40)	0.0296	2.1926
Q effluent	−(80)	0.0125	0.0607	−(80)	0.0044	0.3259
JZ influent	+(40)	0.0256	0.1243	+(40)	0.0230	1.7037
JZ effluent	+(40)	0.0335	0.1627	+(80)	0.0125	0.9259

^a Positive/negative: “+”—positive; “−”—negative; the value in bracket represents the first volume (mL) or concentration (μg/mL) detected showing positive response or the maximum volume with negative result

G, J and Q RWTPs are located in Beijing and JZ RWTP is located in Tianjin

The performance of the two extraction reagents in detecting *umuC* gene expressions induced by environmental samples in the presence and absence of S9 mix was further compared as shown in Table 2. Among the twenty-seven samples, twelve samples (two drinking water, nine reclaimed water, and one wastewater samples) elicited positive responses when using the B. M. Mix treatment, however, only three samples (two reclaimed water and one wastewater samples) showed positive responses using the SDS treatment. The three positive responses observed in the *umu* test using the SDS extraction system were also caught in that using the B. M. Mix system. At the same time, the sample volumes required for the positive results were lower in the B. M. Mix treated tests (Table 2). It should be noted that one drinking water sample (D10) exhibited a high carcinogenic risk, which was over the control standard (10^{-6}) recommended by EPA (2006). On the other hand, even for those without positive response, such as D3–D8 (drinking water), R2 and R3 (reclaimed water), a linear dose–response relationship between the genotoxic induction ratios and their corresponding original volumes was observed in the tests using B. M. Mix, making it possible to calculate the 4-NQO or 2-AA equivalents of the samples (Table 2), which is helpful for the risk management of drinking water (Zhu et al. 2008) and in comparing the changes in genotoxicity of reclaimed water (Zhang and Zhao 2013). Both the assays of genotoxins and water samples showed B. M. Mix could increase the sensitivity of *umu* test. In addition to the difference of β -galactosidase's stability in both reagents, such an improvement could be further explained by the difference in cell lytic ability between the two systems. According to a previous study (De Mey et al. 2008), the use of SDS could result in poor protein recovery, while B. M. Mix was found to be one of the most reliable methods for extracting protein

from *E. coli* whole cells and was further recommended as the cell disruption method for proteomic analysis of *Paenibacillus sp.* (Ganesh and Lin 2011; Chambers 2002).

As an application, the genotoxicity of the influent and effluent of four reclaimed water treatment plants (RWTPs) was evaluated using B. M. Mix as the lytic reagent. The influent of G RWTP exhibited the highest genotoxicity, whereas the effluent of Q RWTP the lowest without S9 activation. Seven of the eight samples, particularly the three effluent samples, exhibited decreased SOS-induction activity (SOSIA) values under the addition of S9 mix, showing that the genotoxins in these samples could be inactivated by S9 enzymes. Of the four RWTPs investigated, G plant, using the traditional coagulation-sand filtration technology, showed the lowest efficiency in removing the genotoxicity from its influent, while both Q and J plants, applying ozonation, exhibited satisfactory genotoxicity removal performance. Ozone as a strong oxidant has exhibited high efficiency in decreasing genotoxicity from wastewater in previous studies (Misik et al. 2011; Cao et al. 2009). The coagulation-microfiltration-chlorination process adopted by JZ RWTP could effectively remove UV_{254} and DOC (data not shown), but the genotoxic potential in its final effluent increased by 30.86 % compared with that of the influent (Table 3). The increased genotoxicity might be related with the high residual chlorine in the effluent (0.57 mg/L). In fact, chlorination disinfection byproducts, such as chlorinated aromatic hydrocarbons and halogenated alkanes, have been reported to contribute to the increase of genotoxicity after chlorination (Shen et al. 2003).

In conclusion, comparing with the conventional reagent, B. M. Mix could maintain the stability of β -galactosidase, which was favorable for ensuring measurement accuracy.

More important, the sensitivity of the *umu*-microplate test could be markedly improved by adopting B. M. Mix as the enzyme extraction reagent. Ozonation was found to be effective in removing genotoxicity from wastewater, while chlorination of the reclaimed water led to the increase of genotoxicity. Thus, careful consideration should be taken in choosing the treatment and disinfection technologies for wastewater reclamation to reduce environmental risks.

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Conflict of interest None declared.

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