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Development of an indirect competitive immunoassay for determination of L-hydroxyproline in milk

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L-hydroxyproline can be used as a marker for hydrolysed leather protein. This is the first study reporting an indirect competitive enzyme-linked immunosorbent assay (ELISA) for determination of L-hydroxyproline in milk. A hapten for L-hydroxyproline was synthesised and used to produce a monoclonal antibody. The antibody was specific to the hapten and showed low crossreactivity to parent L-hydroxyproline. After evaluation of different coating antigen/antibody combinations, a heterologous ELISA was developed to determine L-hydroxyproline in milk. Milk samples were hydrolysed with concentrated sulfuric acid in microwave oven to release free L-hydroxyproline that was derivatised with *p*-hydroxybenzaldehyde. The *p*-hydroxyphenyl L-hydroxyproline was determined by the ELISA, and the limit of detection calculated as L-hydroxyproline was 0.04 µg/mL. The recoveries of L-hydroxyproline from fortified milk ranged from 88.6% to 102.5%, with coefficients of variation ranging from 4.1% to 9.7%. Therefore, ELISA could be used as a rapid method to monitor the presence of L-hydroxyproline in milk.

Keywords: L-hydroxyproline; *p*-hydroxybenzaldehyde; monoclonal antibody; ELISA; milk

1. Introduction

Hydrolysed animal protein (HAP), such as fish hydrolysate (Kristinsson & Rasco, 2000), is a class of special protein from hydrolysis of animal fur, hair or bone. The main ingredient of HAP is collagen that can be used as nutrient and replenisher in special diets for the patients who are unable to take ordinary food protein. Therefore, HAP is good for the health of human beings.

The cost for production of edible HAP is high, so the addition of high-cost HAP into common foods (e.g. packaged milk) would not bring the desirable profit to the milk producers. In the last few years, some milk producers in China illegally added the simply hydrolysed solid leather wastes into milk with the aim to increase protein content, decrease production cost and gain high profit. This kind of product was called 'leather-derived HAP'. Though this product contained HAP, the commonly used potassium dichromate and sodium dichromate in leather manufacture were also added

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into milk at the same time. Long-term consumption of chromium-containing milk is toxic to the consumers, so the Ministry of Health of China has prohibited the addition of 'leather-derived HAP' into foodstuffs since 2011. Therefore, it is very important to monitor the presence of 'leather-derived HAP' in milk. However, there has been no official method issued for the determination of 'leather-derived HAP' in foods so far.

The amino acids in HAP are different from that in milk proteins (e.g. lactoglobulin and lactalbumin), because HAP contains an unusual amino acid, L-hydroxyproline (HP, Figure 1), which is absent in other proteins (Miller, Martin, & Piez, 1964). If HP is found in a milk sample, then the sample can be identified as the 'leather-derived HAP-added milk'. According to this conception, some methods have been developed to determine HP in various foods. In many methods, HP was derivatised with chloramine T and 4-dimethylaminobenzaldehyde to form a red derivative that was then determined by ultraviolet (UV)-Visible spectrophotometer at 558 nm (China standard method, 2008; Li, Yang, & Meng, 2007; Liu & Cao, 2009; Reddy & Enweremeka, 1996; Tian, 2008; Xu, Zhou, & Shi, 2005). In other methods, HP was derivatised with AccQ-Fluor reagent (Jin, Lin, Zhao, Zhu, & Shi, 2009), 2,4-dinitrofluorobenzene (Hu, Lv, Du, Zhang, & Zhu, 2010) or phenyl isothiocyanate (Cui, Liu, Zhang, Jiang, & Li, 2008), and the derivatives were determined by high performance liquid chromatography (HPLC) method. Besides, HP was also determined by liquid chromatography mass spectrometry (Sun, Lu, Ma, Cao, & Zhao, 2007; Xia, Chen, & Yao, 2008) and other methods (Ding et al., 2011; Etherington & Trevor, 1981). The limits of detection (LOD) of these methods for HP were in the range of 0.7 ng/mL to 2.5 µg/mL.

Compared with those methods, enzyme-linked immunosorbent assay (ELISA) is a rapid, low-cost and sensitive method capable of screening large numbers of samples in a single test. However, there has been no paper reporting ELISA method for the detection of HP in foods so far. For the development of an ELISA method, the first thing is to produce the antibody against the analyte. In our recent study, a hapten of

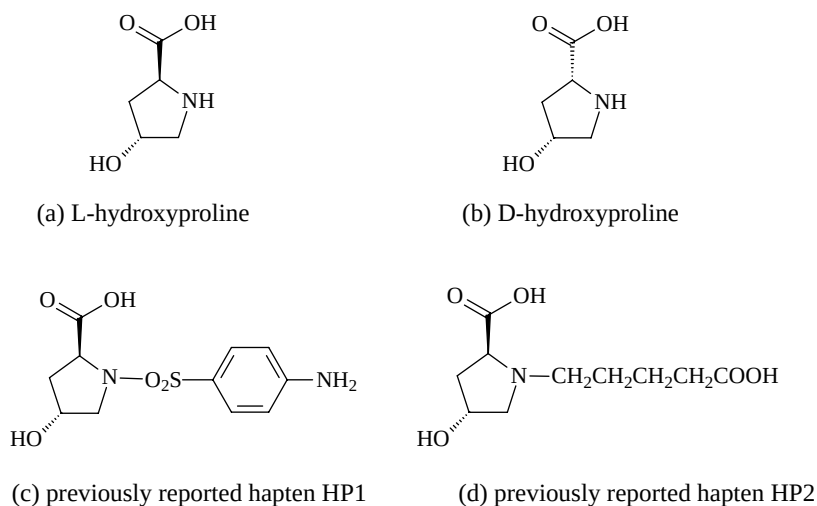


Figure 1. Chemical structures of (a) L-hydroxyproline, (b) D-hydroxyproline, the previous haptens (c) HP1 and (d) HP2.

HP (HP1, Figure 1) was synthesised and used to produce the monoclonal antibody, but the obtained antibody showed high specificity to HP1 and showed low crossreactivity (CR) to the parent HP (Wang, Liu, Liu, & Wang, 2013).

The chemical structure of HP is very simple and its general structure is similar to the structure of furazolidone metabolite (AOZ). In the previous reports, AOZ was derivatised with 3-carboxybenzaldehyde to synthesise the hapten that was used to produce the antibodies, but the obtained antibodies showed high specificity to the hapten and showed negligible CR to parent AOZ (Cooper, Caddell, Elliott, & Kennedy, 2004; Diblikova, Cooper, Kennedy, & Franek, 2005). Therefore, AOZ was derivatised with 2-nitrobenzaldehyde to form a derivative that was then determined by using the antibodies, and the AOZ concentration was calculated through the derivative according to the ratio of molecular weights between the derivative and AOZ. Therefore, it seemed possible to develop an ELISA method for determination of HP according to the methods for determination of AOZ. In the present study, a new monoclonal antibody against HP was produced that was used to develop a heterologous ELISA for the determination of HP in milk.

2. Materials and methods

2.1. Reagents and materials

HP, D-hydroxyproline, bovine serum albumin (BSA) and ovalbumin (OA) were purchased from Sigma (St. Louis, MO). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). *p*-Hydroxybenzaldehyde and other chemical reagents were all analytical grade or better from Beijing Chemical Company (Beijing, China).

PBS (pH 7.2) was prepared by dissolving 0.2 g KH_2PO_4 , 0.2 g KCl, 1.15 g Na_2HPO_4 and 8.0 g NaCl in 1000 mL deionised water. Washing buffer (phosphate-buffered saline with Tween 20 (PBST) was PBS buffer containing 0.05% Tween. Coating buffer was sodium carbonate buffer (0.1 M, pH 9.6). Substrate buffer was 0.1 M sodium citrate (pH 5.5). The substrate system was prepared by adding 200 μL 1% (w/v) TMB in DMSO and 64 μL 0.75% (w/v) H_2O_2 into 20 mL substrate buffer.

2.2. Synthesis of hapten helix pomatia hemocyanine

The synthetic route of the hapten is shown in Figure 2. Briefly, 130 mg HP and 122 mg *p*-hydroxybenzaldehyde were dissolved in 5 mL of ethanol. Then 200 μL of acetic acid was added, and the mixture was refluxed for 2 hours under heat to obtain some sediment. Finally, the suspension was filtered under vacuum and the deposit was washed with 20 mL of ethanol, and subsequently dried to yield the hapten helix pomatia hemocyanine (HPH) (melting point 245°C ; IR (KBr) ν_{max} 3284, 3300-2450, 3137, 2950, 2732, 1639, 1600, 1480, 1400, 1321, 1068, 962 cm^{-1}).

2.3. Preparation of the conjugates

The hapten was coupled to carrier protein by use of *N,N'*-carbonyldiimidazole (Figure 2). Briefly, 25 mg HPH and 81 mg *N,N'*-carbonyldiimidazole were added into 5 mL of acetone to be stirred for 4 hours. Then the solution was added dropwise into

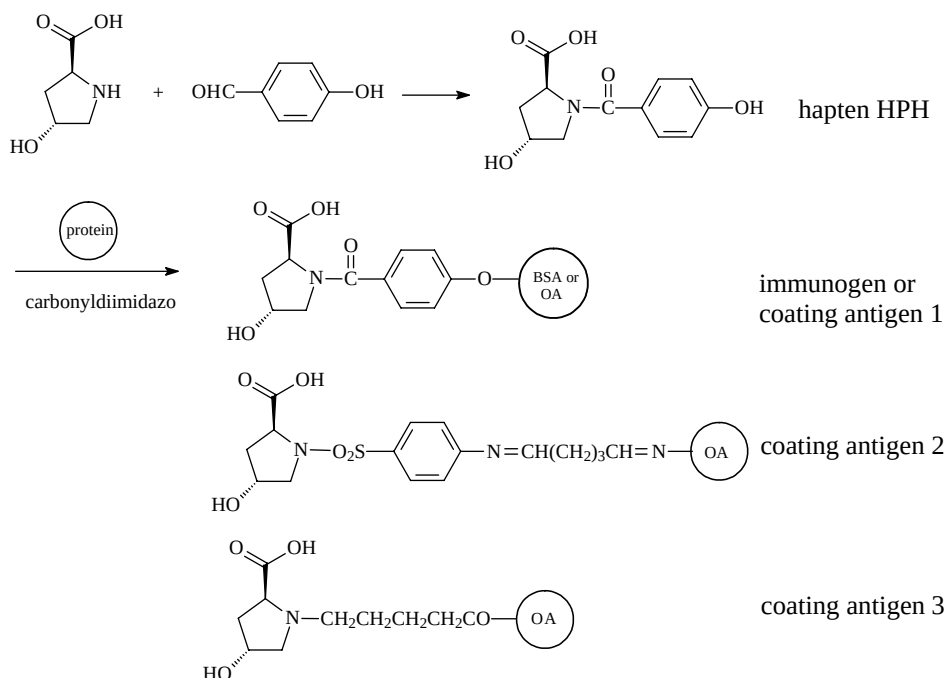


Figure 2. Synthetic processes of the hapten HPH and the used conjugates.

a PBS solution containing 136 mg BSA or 60 mg OA. The mixture was stirred for 12 hours to prepare the immunogen (HPH-BSA) or the coating antigen (HPH-OA). The conjugates were purified by passing through a home-made Sephadex G25 cartridge, and the collected eluate were dialysed against PBS for 3 days at 4°C. HPH, the carriers and the conjugates were all scanned on a UV spectrophotometer to verify the conjugation. The hapten/carrier coupling ratios were calculated according to the previous 2,4,6-trinitrobenzene sulfonic acid method (Sashidhar, Capoor, & Ramana, 1994).

2.4. Production of the monoclonal antibody

Five eight-week-old female BALB/c mice were immunised subcutaneously with an emulsion of the immunogen (50 µg per animal, calculated as protein) in Freund's complete adjuvant in the dorsal region. The mice were boosted with an emulsion of the immunogen in Freund's incomplete adjuvant at two-week intervals. After seven boosters, the mice were immunised with a final dose of the immunogen in sterilised normal saline. Through the immunisations, the sera were collected and the antibody titers were monitored. The spleen from the mouse with the highest titer was removed and the splenocytes were fused with SP2/O myeloma cells. The splenocyte fused myeloma cells were cultured in 96-well plates and the positive hybridomas were screened by using the indirect competitive ELISA described below with HPH as the competitor. The hybridomas producing the specific monoclonal antibody to HPH were sub-cloned twice with the limiting dilution method, and the sub-cloned

hybridoma cells were collected and frozen in liquid nitrogen. The ascites from hybridoma-induced mice were purified by using the saturated ammonium sulfate precipitation method to obtain the monoclonal antibody.

2.5. Development of the ELISA

In this study, two coating antigens previously prepared in our lab (coating antigen 2 and 3, Figure 2) (Wang et al., 2013) and HPH-OA (coating antigen 1) were used to evaluate the monoclonal antibody. A checkerboard procedure was used to determine the optimal dilutions of coating antigen and antibody, in which the well with an absorbance (OD) of 1.0 was defined as the optimal dilutions of the coating antigen and the antibody. After that, each well of a microtiter plate was coated with 100 μ L of coating antigen, incubated overnight at 4°C, and then blocked with 1% fetal calf serum. The plate was washed three times with PBST. Then 50 μ L of the optimal antibody dilution and 50 μ L of HPH solution with series concentrations were added into the wells (in triplicate) for incubation for 1 hour at 37°C. The plate was washed as above. Then, 100 μ L of horseradish peroxidase-labeled goat anti-mouse immunoglobulin G was added for incubation for 30 minutes at 37°C. After washes, 100 μ L of TMB substrate system was added for incubation for 15 minutes at 37°C. Finally, the reaction was stopped by addition of 50 μ L of 2 M H₂SO₄, and the plate was read on an ELISA plate reader at 450 nm to obtain the OD of each well.

D-hydroxyproline, 5-hydroxylysine, proline, *N*-acetylsulfanilyl chloride and *p*-hydroxybenzaldehyde were also determined as described above. The half-inhibition concentrations (IC₅₀) and the LOD for these competitors were determined as the concentrations showing 50% and 10% of inhibition, respectively. The competitive inhibitory curve was developed by plotting the concentrations (Log C) versus the *B/B*₀ values (mean OD value of the HPH solutions divided by that of zero standard). The CR was calculated as follows:

$$CR (\%) = \frac{IC_{50 \text{ HPH}}}{IC_{50 \text{ competitor}}} \times 100.$$

2.6. Sample Preparation

A volume of 1 mL of milk and 6 mL of 6 mol/L H₂SO₄ were added into a conical flask and stirred vigorously for 2 minutes. Then the flask was heated for 8 minutes in a microwave oven at high power (about 700 W). The above procedures were used to hydrolyse HAP and release free HP according to a previous report (Ding et al., 2011). After cooling down to room temperature, the pH of the mixture was adjusted to 6.5 with 12 mol/L NaOH. Then 1 mL of ethanol containing 10 mg *p*-hydroxybenzaldehyde was added, and the mixture was stirred in a water bath (80°C) for 2 hours. After cooling down to room temperature, the mixture was centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and filtered with a 0.45 μ m Millipore filter for ELISA analysis.

Milk samples obtained from several controlled farms were used as the blank samples. The extracts from the blank milk were used to prepare the matrix-matched HPH solutions (0.024, 0.048, 0.096, 0.19, 0.38, 0.96, 1.92, 3.85, 9.62 μ g/mL).

The ratio of molecular weights between HPH and HP was 1.92, so these concentrations as HP equivalents were 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 µg/mL, respectively. The blank milk samples were fortified with HP standard at concentrations of 0.5, 2.0, 10.0 and 40.0 µg/mL to evaluate the accuracy (intra- and inter-assay recovery) and the precision. The intra-assay recoveries were calculated from the results of six replicates in a single day and the inter-assay recoveries were calculated from the results on six successive days. The precision was expressed as coefficient of variation. Thirty-five unknown commercial packaged milk samples from several supermarkets in China were analysed by the developed method.

3. Results and discussions

3.1. Hapten and immunogen

The chemical structures of nitrofurantol metabolites are simple. Therefore, nitrofurantol metabolites were all derivatised with 3(4)-carboxybenzaldehyde to synthesise the haptens that were used to produce the antibodies in the previous reports (Chang, Peng, Wu, Wang, & Yuan, 2008; Cooper et al., 2004; Diblikova et al., 2005; Gao, Chen, Cheng, Lei, & Zeng, 2007; Lui et al., 2007; Pimpitak, Putong, Komolpis, Petsom, & Palaga, 2009; Vass, Diblikova, & Franek, 2008). HP also contains a simple molecular structure (Figure 1). In our previous study, two haptens of HP were synthesised (HP1 and HP2, Figure 1) and only HP1 stimulated the animal immune system generating the antibody (Wang et al., 2013). However, the resultant monoclonal antibody was highly specific to HP1 but showed low CR to parent HP (Table 1).

Table 1. Performance of antibody HPH-5M7 and the previously reported anti-HP1 antibody when using different coating antigens.

Analyte	HPH-OA		Coating antigen 2		Coating antigen 3	
	IC ₅₀ ^a	CR ^b	IC ₅₀	CR	IC ₅₀	CR
antibody HPH-5M7						
HPH	0.44	100	0.39	100	0.28	100
HP1	0.54	81	0.47	83	0.3	91
HP2	14.6	3	7.8	5	3.5	8
L-hydroxyproline	7.3	6	4.3	9	1.65	17
D-hydroxyproline	>44	<1	>39	<1	28	1
Previously reported anti-HP1 antibody						
HPH	0.26	82	0.31	80	0.18	87
HP1	0.21	100	0.25	100	0.16	100
HP2	>21	<1	>25	<1	>16	<1
L-hydroxyproline	1.5	14	1.85	13	0.89	18
D-hydroxyproline	10.5	2	23	1.1	8	2.0

^aThe units of IC₅₀ and LOD are µg/mL.

^bCR is expressed as %.

In the present study, HP was derivatised with *p*-hydroxybenzaldehyde to synthesise a new hapten HPH (Figure 2). First, the melting point of HPH (245°C) was different from that of HP (274°C) and *p*-hydroxybenzaldehyde (117°C), indicating a new compound was obtained. Second, the infrared data showed HPH contained a benzene ring and a –CO – N bond besides the original chemical groups of HP, indicating the general structure of HP was present. Third, the UV spectrum of HPH (Figure 3) indicated that *p*-hydroxybenzaldehyde was coupled with HP (HP itself has no UV absorption, Figure 3). These data were able to prove the designed hapten was obtained.

Then, the hapten was coupled to carrier protein by use of *N,N'*-carbonyldiimidazole method (Figure 2). There were two hydroxyl groups in the molecule of HPH; the newly introduced phenol hydroxyl group was far from the HP moiety and its reactivity was higher than the original hydroxyl group in HP, so the hapten/carrier coupling position should be at the newly introduced phenol hydroxyl group. The UV spectrum of HPH-BSA contained the characteristic peaks of HPH and BSA (Figure 3), indicating the successful conjugation. The hapten density was 14 mol/mol in HPH-BSA and 12 mol/mol in HPH-OA. These coupling ratios, calculated

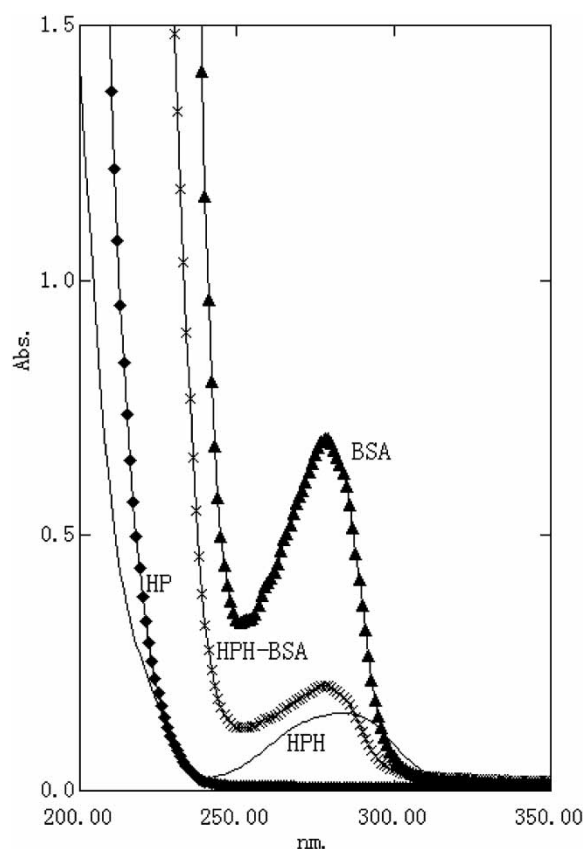


Figure 3. UV scan spectra of hapten HP, HPH, BSA and HPH-BSA.

according to the previous method (Sashidhar et al., 1994), also indicated the successful conjugation.

3.2. Performances of the monoclonal antibody

Berek and Milstein showed the repeated immunisation could induce somatic hypermutation within antigen-stimulated B cells further to enhance the affinity of the resulting antibody (Berek & Milstein, 1987). In the present study, the mice were immunised nine times in order to produce the sensitive antibody. Three hybridomas producing the specific antibody to HPH were obtained (HPH-1M3, HPH-5M7, HPH-6M24), and the most sensitive antibody HPH-5M7 was selected for the subsequent experiments. The specificity and sensitivity of the antibody to the competitors are shown in Table 1.

As shown in Table 1, antibody HPH-5M7 was highly specific for HPH (IC_{50} 0.44 μ g/mL, equivalent of HP 0.23 μ g/mL) and showed low CR (6%) to parent HP. This finding was similar to our previous result (Wang et al., 2013). Furthermore, the antibody showed high CR (81%) to the previous hapten HP1 (IC_{50} of 0.54 μ g/mL, equivalent of HP 0.24 μ g/mL) but showed low CR (3%) to the previous hapten HP2. This was because the general structure of HP1 was similar to HPH (Figures 1 and 2). As expected, the antibody showed negligible CRs to D-hydroxyproline (<1%) and other competitors (data not shown). The previous reports showed the antibody binding for its analyte was based on the three-dimensional recognition (Adrian, Font, Diserens, Sánchez-Baeza, & Marco, 2009; Greirson, Allen, Gare, & Waston, 1991). Though the molecular formulae of D-hydroxyproline and HP are same, their three dimensional structures are different. Therefore, the antibody showed negligible CR to D-hydroxyproline.

3.3. Heterologous ELISA

Heterology in the coating antigen is commonly used to improve an immunoassay (Adrian et al., 2009; Franek et al., 2001; Goodrow & Hammock, 1998; Greirson et al., 1991; Liu, Zhang, Zhang, Gao, & Wang, 2012; Xu et al., 2010). The heterologous hapten can eliminate the affinity of the antibody from the space arm in the immunogen that leads to no or poor inhibition by the competitor (Greirson et al., 1991). In this study, the previous two coating antigens and anti-HP1 monoclonal antibody (Wang et al., 2013), coating antigen 1 HPH-OA and antibody HPH-5M7 were arranged into six combinations to optimise the best reagents combination (Table 1).

As shown in Table 1, the performance of antibody HPH-5M7 for HPH, HP1, HP2 and HP when using coating antigen 2 and 3 (IC_{50} of 0.28–7.8 μ g/mL and CRs of 5%–100%) was better than that when using coating antigen HPH-OA (IC_{50} of 0.44–14.6 μ g/mL and CRs of 3%–100%), demonstrating the advantage of heterologous ELISA. Moreover, the performance when using coating antigen 3 (IC_{50} of 0.28–3.5 μ g/mL and CRs of 8%–100%) was better than that when using coating antigen 2 (IC_{50} of 0.39–7.8 μ g/mL and CRs of 5%–100%). Goodrow and Hammock showed that the better assay sensitivity was obtained when the antibody affinity for the analyte was greater than that for the coating hapten (Goodrow & Hammock,

1998). Moreover, Xu et al. (2010) reported that using a partial structure of the target molecule (or immunising hapten) as the coating hapten was a good strategy. As shown in Figure 2, the coating hapten in coating antigen 3 was a part of the coating hapten in coating antigen 2. This makes the antibody show higher affinity for coating antigen 2 than for coating antigen 3, resulting in better performance when using coating antigen 3.

In our recent study, the HP1 antibody combining coating antigen 3 achieved high sensitivity to HP1 (IC_{50} of 0.16 $\mu\text{g/mL}$, equivalent of HP 0.07 $\mu\text{g/mL}$) (Wang et al., 2013). In the present study, the performance of the previous HP1 antibody when using HPH-OA (heterologous ELISA) was better than that when using coating antigen 2 (homologous ELISA), but worse than that when using coating antigen 3 (partial coating hapten). As shown in Table 1, the previous HP1 antibody always showed high CRs to HPH (80%–87%) with IC_{50} of 0.18–0.31 $\mu\text{g/mL}$ no matter which coating antigen was used. This was because the molecular structures of HPH and HP1 were similar.

The data shown in Table 1 indicated antibody HPH-5M7 and the previous HP1 antibody all showed low sensitivity to parent HP. This meant it was impossible to determine HP directly. However, it was feasible to determine HP by using the two antibodies if HP was derivatised with an appropriate derivatisation reagent, just as the immunoassays of nitrofurantoin metabolites (Chang et al., 2008; Cooper et al., 2004; Diblikova et al., 2005; Gao et al., 2007; Lui et al., 2007; Pimpitak et al., 2009; Vass et al., 2008). As shown in Table 1, the previous HP1 antibody was more sensitive than antibody HPH-5M7. This was because there was a long spacer arm between HP1 and the carrier, whereas there was a short spacer arm between HPH and the carrier. Therefore, the previous HP1 antibody and coating antigen 3 were used for the subsequent experiments.

3.4. Sample preparation and ELISA determination

For analysis of HP, the first step was to hydrolyse HAP and release free HP. In the previous reports, free HP was usually released by hydrolysis of HAP with concentrated hydrochloric acid (6 mol/L) at 110°C for 12 hours (China standard method, 2008; Cui et al., 2008; Etherington & Trevor, 1981; Hu et al., 2010; Jin et al., 2009; Li et al., 2007; Liu & Cao, 2009; Reddy & Enwermeka, 1996; Sun et al., 2007; Tian, 2008; Xia et al., 2008; Xu et al., 2005). Recently, Ding et al. (2011) reported a microwave-assisted acid hydrolysis method to release HP. The sample preparation time was short and the HAP hydrolysis degree was similar to other reported methods. Therefore, this method was used in the present study to hydrolyse HAP and release free HP.

Because the previous HP1 antibody showed low sensitivity to parent HP, an appropriate derivatisation step was required. During the experiments, *p*-hydroxybenzaldehyde, *N*-acetylsulfanilyl chloride, *p*-carboxybenzaldehyde and *p*-aminobenzaldehyde were tested to optimise the best derivatisation reagent. After evaluation of reaction time, simplicity, operability of the derivatisation method and the IC_{50} value, *p*-hydroxybenzaldehyde was selected as the optimal derivatisation reagent.

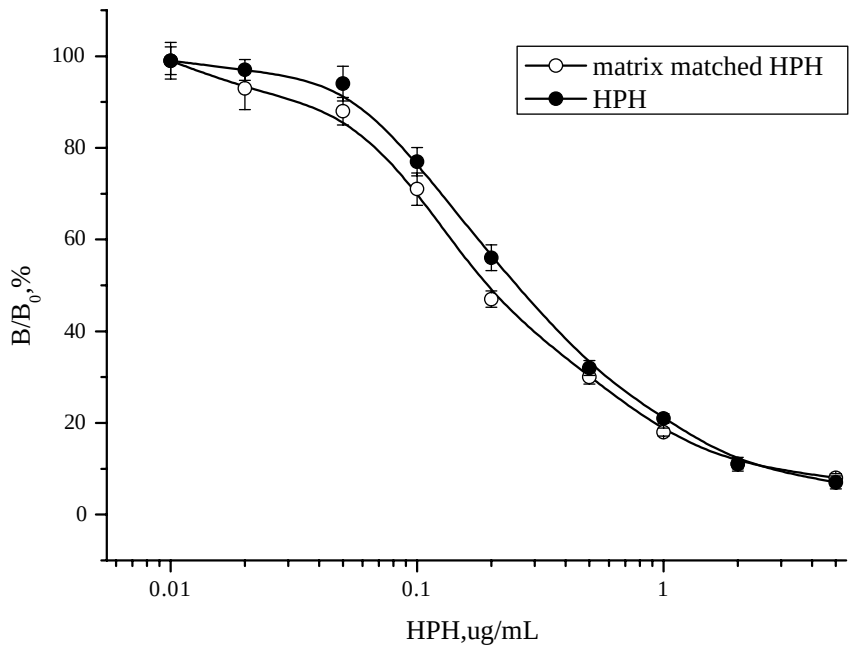


Figure 4. Competitive inhibitory curve of HPH standard and matrix matched HPH.

HPH was diluted with the blank extracts to prepare the matrix-matched HPH solutions. As shown in Figure 4, the competitive inhibitory curve of matrix-matched HPH was similar to that of HPH standard, indicating the sample preparation was satisfactory. The LOD for HPH was $0.08 \mu\text{g/mL}$, and this value as HP equivalent was $0.04 \mu\text{g/mL}$. The sensitivity of the ELISA method was higher than the spectrophotometer and HPLC methods (China standard method, 2008; Cui et al., 2008; Hu et al., 2010; Jin et al., 2009; Li et al., 2007; Liu & Cao, 2009; Reddy & Enweremeka, 1996; Tian, 2008; Xu et al., 2005), but lower than the liquid chromatography-electrospray ionization-mass/mass spectrometry method (Sun et al., 2007; Xia et al., 2008). Then, the blank milk samples were fortified with parent HP at four levels to evaluate the accuracy and the precision. The intra- and inter-assay recoveries were in the range of 88.6% to 102.5%, with coefficients of variation lower than 9.7% (Table 2). The 35 unknown milk samples were all analysed by the developed ELISA method, but no sample was determined as positive.

Table 2. Intra- and inter-assay recoveries of parent HP from blank fortified milk.

Added ($\mu\text{g/mL}$)	Intra-assay			Inter-assay		
	Detected ($\mu\text{g/mL}$)	Recovery (%)	CV (%)	Detected ($\mu\text{g/mL}$)	Recovery (%)	CV (%)
0.5	0.45 ± 0.03	90.2	6.8	0.44 ± 0.05	88.6	9.4
2.0	1.96 ± 0.08	98.4	4.1	1.92 ± 0.1	96.2	6.0
10	9.73 ± 0.5	97.3	5.0	10.3 ± 0.7	102.5	6.7
40	39.5 ± 3.2	98.7	8.3	3.86 ± 3.9	96.4	9.7

4. Conclusion

As a class of special protein, HAP has no harmful effect to human being. However, the simply treated leather wastes containing heavy metal ions are toxic to the consumers. Therefore, China government has forbidden the addition of leather-derived HAP into milk. For inspection of the HAP from leather wastes, a special amino acid, HP, can be used as the target analyte. The present study is the first report of a heterologous ELISA method to determine HP in milk by using a monoclonal antibody of HP derivative. Milk sample was hydrolysed with sulfuric acid under the assistance of microwave, and the released parent HP was derivatised with *p*-hydroxybenzaldehyde for ELISA analysis. The LOD, calculated as parent HP, was 0.04 µg/mL. Therefore, this ELISA procedure could be used as a rapid screening method to monitor the HAP from leather wastes in milk with HP as the target analyte.

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