UDC 577.156.2

APPLICATION OF STANDARD ADDITION FOR THE DETERMINATION OF CARBOXYPEPTIDASE ACTIVITY IN *Actinomucor elegans* BRAN KOJI

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Received December 15, 2010

Leucine carboxypeptidase (EC 3.4.16) activity in *Actinomucor elegans* bran koji was investigated via absorbance at 507 nm after stained by Cd-nihydrin solution, with calibration curve A, which was made by a set of known concentration standard leucine, calibration B, which was made by three sets of known concentration standard leucine solutions with the addition of three concentrations inactive crude enzyme extract, and calibration C, which was made by three sets of known concentration standard leucine solutions with the addition of three concentrations crude enzyme extract. The results indicated that application of pure amino acid standard curve was not a suitable way to determine carboxypeptidase in complicate mixture, and it probably led to overestimated carboxypeptidase activity. It was found that addition of crude exact into pure amino acid standard curve had a significant difference from pure amino acid standard curve method ($p < 0.05$). There was no significant enzyme activity difference ($p > 0.05$) between addition of active crude exact and addition of inactive crude kind, when the proper dilute multiple was used. It was concluded that the addition of crude enzyme extract to the calibration was needed to eliminate the interference of free amino acids and related compounds presented in crude enzyme extract.

The basic system for detecting carboxypeptidase activity includes two steps: the first peptide bond from the carbon termini was broken and resulted in the release of a free amino acid. The released free amino acid was detected by colorimetric ninhydrin method, the quantity of the released free amino acids per unit time was then calculated and regarded as carboxypeptidase activity, the more the free amino acids were released, the higher the enzyme activities were obtained.

Most of the compounds with α-NH_2 acid could easily react with ninhydrin. Classic colorimetric ninhydrin methods to detect amino acids and related compounds were developed and Modified by Moore and Stein [1, 2]. There were several modified methods about detection of free amino acids formed during certain reaction, but most of them need special precision instrument such as chromatography [3], electrochemiluminescence [4] or fluorophotometer [5] or some special reagents or substrate [6, 7], couple enzyme [8] or unsafe reducing reagent [9], and some modified method was only limited to assay certain amino acid [10], so the above procedures were cost- and time- consuming to some extent, especially without broad application. On the base of conventional methods, Dio et al. in 1981 reported that modified colorimetric Cd-ninhydrin methods was suitable for the determination of peptidase activities on peptides, because Cd-ninhydrin was more sensitive to and selective for amino acids, comparing with peptides [11]. To date, several explorations have been performed to assay the formation of free amino acids and to assay the carboxypeptidase activity during cheeses proteolysis using modified Cd-ninhydrin method [12–14]. However, application of modified Cd-ninhydrin method was still not capable to assure sensitive or objective for the assay of certain carboxypeptidase in crude extract without purification for there were large amount of free ammonia, primary and secondary amines of protein, peptides and free amino acids presented in the crude enzyme extract which came from the culture media such as bran koji and cheese, and consequently interfered with the detection of the free amino acid generated by the action of carboxypeptidase on specific substrate peptide. Therefore, there was still no precise method for the determination of carboxypeptidase activity in crude microbial culture.

Carboxypeptidase (EC 3.4.16) is a useful tool to eliminate the bitterness presented in protein hydrolysate or some fermented foods which were mainly derived from soybean protein [15–17] and casein [18]. On our previous research, we found that the bitterness of the soybean protein hydrolysate was significantly decreased after treatment of *Actinomucor elegans* extract, which had the carboxypeptidase activity [19]. In order to explore the role of the carboxypeptidase from this mucor in the bitterness decreasing function and the characteristic of the enzyme, it was of great importance that the carboxypeptidase activity assay method

should be objective and accurate. However, complicated compounds presented in crude enzyme extract would give high color yield background according to our preliminary experiment.

Standard addition was frequently used in atomic absorption spectroscopy [20] and gas chromatography [21] and can be applied to most analytical techniques to solve the matrix effect problem [22]. On present research, several sets of leucine calibration curves with the addition of crude enzyme solution were made to assay carboxypeptidase activity in *Actinomucor elegans* crude enzyme extract to meet the demand of analytic precise.

MATERIALS AND METHODS

Reagents. Z-Trp-Leu (N-Carbobenzoxy-Trp-Leu) was purchased from Bachem Inc., (USA); Z-Ala-Leu (N-Carbobenzoxy-Ala-Leu), Z-Phe-Leu (N-Carbobenzoxy-Phe-Leu) and ninhydrin were obtained from Sigma Chemical Co. (USA); L-leucine was purchased from Beijing Dingguo Biotechnology Co. (China).

Leucine (2.0 mM) in distilled water was served as standard amino acid solution in this experiment. Substrates of Z-Trp-Leu, Z-Ala-Leu and Z-Phe-Leu were dissolved in pH 7.0, 0.1 M/l potassium phosphate buffer to a final concentration of 2.0 mM/l. 0.8 g ninhydrin was dissolved in a mixture of 80 ml of 99.5% ethanol and 10 ml of acetic acid, and 1.0 g $CdCl_2$ was dissolved in 1 ml water, after both of the reagent were fully dissolved, they were mixed and kept in brown reagent bottle at room temperature [9].

Sample. *Culture and culture conditions. Actinomucor elegans* AS3.2778 was generously provided by Wang Zhihe Sufu Co. (Beijing, China). The inoculum culture was prepared by inoculating the *A. elegans* spores with an inoculating needle from a slope culture into the tofu cake medium in a 250 ml flask, which was previously sterilized at 121°C for 15 min. The culture was incubated at 28°C for 72 h. The spore suspension was prepared by suspending the contents of inoculum culture into 100 ml sterile water. Wheat bran (10 g) with water (12 ml) in a 250 ml flask was autoclaved for 45 min at 121 °C. After the spore suspension (2 ml; containing about 3×10^6 viable propagules/ml) was uniformly dispensed into the wheat bran medium in the flasks, the flask containers were then incubated at 28°C for 2 days.

Crude enzyme extract. After incubation, the solid mould medium was mixed thoroughly with 100 ml sterile distilled water and left to soak at 4°C overnight. The fermented extract was then squeezed out through a cheese cloth and centrifuged at 4020 g and 4°C (Hitachi CR 22G, Japan) to remove the insoluble. The clear supernatant of the extract was the crude enzyme extract.

Dilute enzyme solutions with different concentrations for enzyme-substrate reaction. 8.0, 2.0 and 1.0 ml fresh crude enzyme extract was diluted with pH 7.0, 0.1 M potassium phosphate buffer to 10.0 ml in volumetric flasks separately to prepare 1.25, 5 and 10 dilute folds of crude enzyme extract, then half (5 ml) of the dilute enzyme extracts were transferred to another 3 tubes which were then put into boiling water and kept for 5 min to prepare 1.25, 5 and 10 folds inactive dilute crude enzyme extract.

Dilute enzyme extract with different concentrations for calibration curves making. 200.0, 50.0 and 25.0 μl fresh crude enzyme extract was diluted with pH 7.0, 0.1 M potassium phosphate buffer to 10 ml in volumetric flasks separately to prepare 50, 200 and 400 folds dilute crude enzyme extract (the dilute folds were calculated according to the dilute folds for enzyme-substrate reaction), then half (5 ml) of the dilute enzyme extracts were transferred to another 3 tubes which were then put into boiling water and kept for 5 min to prepare 50, 200 and 400 folds inactive dilute crude enzyme extract.

Procedure. *Determination of carboxypeptidase activities.* The reaction mixture, consisting of 1.8 ml substrate solution (2.0 mM) and 0.2 ml diluted crude enzyme extracts which were provided by three dilute solutions: 1.25, 5.0 and 10.0 respectively, was incubated at 37°C for up to 6 h. Aliquots (50.0 μl) were taken and mixed with 950 μl distilled water and 2.0 ml Cd-ninhydrin reagent, then incubated at 84°C for 5 min, amino acids were assayed by measuring the absorbance at 507 nm and calculated according to the calibration curves. The blank consisted of 1 ml H_2O and 2 ml Cd-ninhydrin reagent and monitored under the same incubation conditions. The carboxypeptidase activity was expressed as μmol of leucine h^{-1} ml^{-1} crude enzyme extract.

Preparation of calibration curves. Calibration curve A was made in the conventional standard curve way. 0, 12.5, 25, 37.5, 50, 62.5, 75 μl leucine solution (2.0 mM) was transferred to a set of tubes separately, and mixed with 1000, 987.5, 975, 962.5, 950, 937.5, 925 μl distilled water in turn. The final leucine concentration of the set was 0, 25, 50, 75, 100, 125 and 150 μM which was stained by 2.0 ml Cd-ninhydrin reagent respectively and the absorbance was read at 507 nm. The blank consisted of 1 ml H_2O and 2 ml Cd-ninhydrin reagent. The Calibration curve A was made according to the leucine concentration and absorbance at 507 nm which was showed in Fig. 1.

Calibration curve B was made with the addition of inactive dilute crude enzyme extract. 0, 12.5, 25, 37.5, 50, 62.5, 75 μl leucine solution (2.0 mM) was transferred to a set of tubes separately, then 200 μl of diluted inactive crude enzyme solution was added into each tube, and mixed with 800, 787.5, 775, 762.5, 750, 737.5, 725 μl distilled water in turn. The final Leu concentration of the set was 0, 25, 50, 75, 100, 125 and 150 μmol/l which was stained by 2.0 ml Cd-ninhydrin reagent respectively and the absorbance was read at 507 nm. The blank 1consisted of 1 ml H_2O and 2 ml Cd-ninhydrin reagent. Calibration a, b and c were showed in Fig. 2.

Calibration curve C was made with the addition of active dilute crude enzyme extract. 0, 12.5, 25, 37.5,

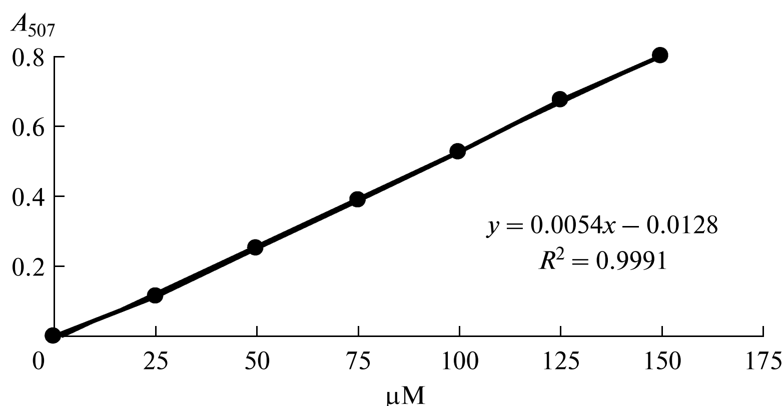

Fig. 1. Calibration curve A: A_{507} vs. leucine (μmol/l).
2.0 mM leucine was diluted to a set of concentration in the range of 0–150.0 μM with distilled water, to 1.0 ml of final volume, then 2.0 ml Cd-ninhydrin reagent was added to the leucine solutions respectively.

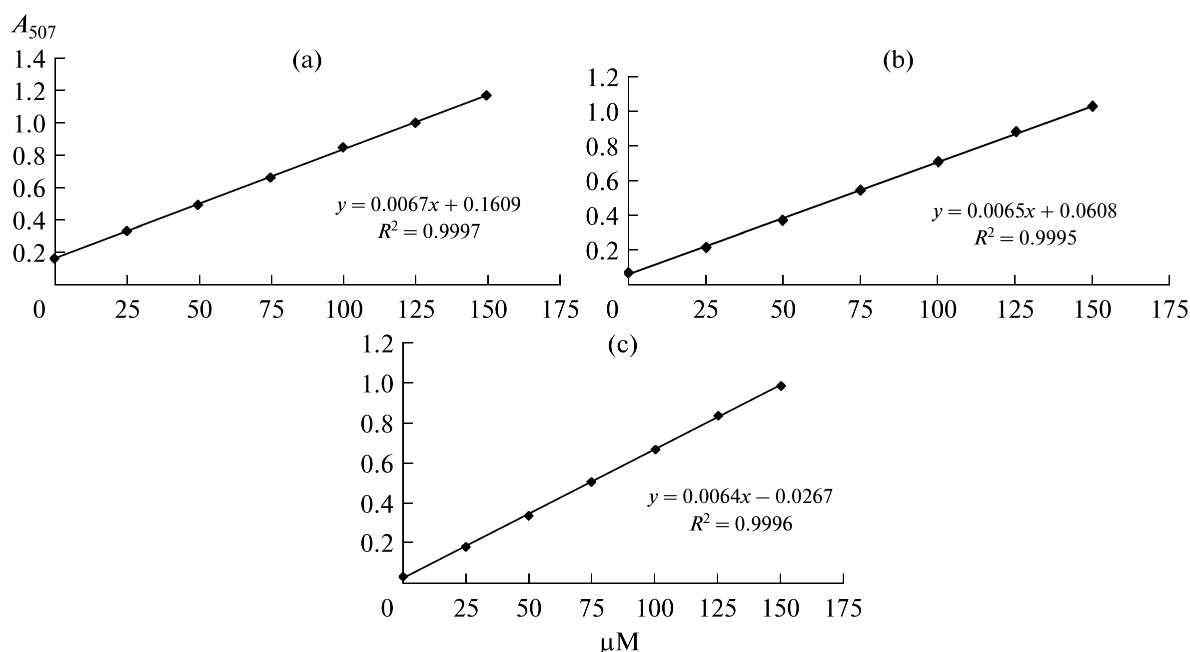

Fig. 2. Calibration curves B: A_{507} vs. leucine (μM) with the addition of inactive crude enzyme.
2.0 mM leucine was diluted to 3 sets of concentration in the range of 0–150.0 μM with 200.0 μl three concentrations dilute inactive crude enzyme extract (diluent fold was 50, 200, or 400 respectively) and distilled water, to 1.0 ml of final volume. Dilute multiple of the inactive crude enzyme extract was 50 (a), 200 (b), 400 (c).

50, 62.5, 75 μl leucine solution (2.0 mmol/l) was transferred to a set of tubes separately, then 200 μl of diluted active crude enzyme solution was added into each tube, and mixed with 800, 787.5, 775, 762.5, 750, 737.5, 725 μl distilled water in turn. The final Leu concentration of the set was 0, 25, 50, 75, 100, 125 and 150 μmol/l which was stained by 2.0 ml Cd-ninhydrin reagent respectively and the absorbance was read at 507 nm. The blank consisted of 1 ml H_2O and 2 ml Cd-ninhydrin reagent. (As on Figs. 1 and 2). Calibration C (a–c) were showed in Fig. 3.

Statistical analysis. All the tests were done 3 times and the data were averaged. Standard deviation was also calculated. SAS V.9.0 was used to evaluate significantly different ($p < 0.05$) means for each sample.

RESULTS AND DISCUSSION

Differences among Calibration curve A, calibration curve B and calibration curve C. Figure 1 showed the calibration curve A which was the general linear relationship between leucine concentration and absorbance, the

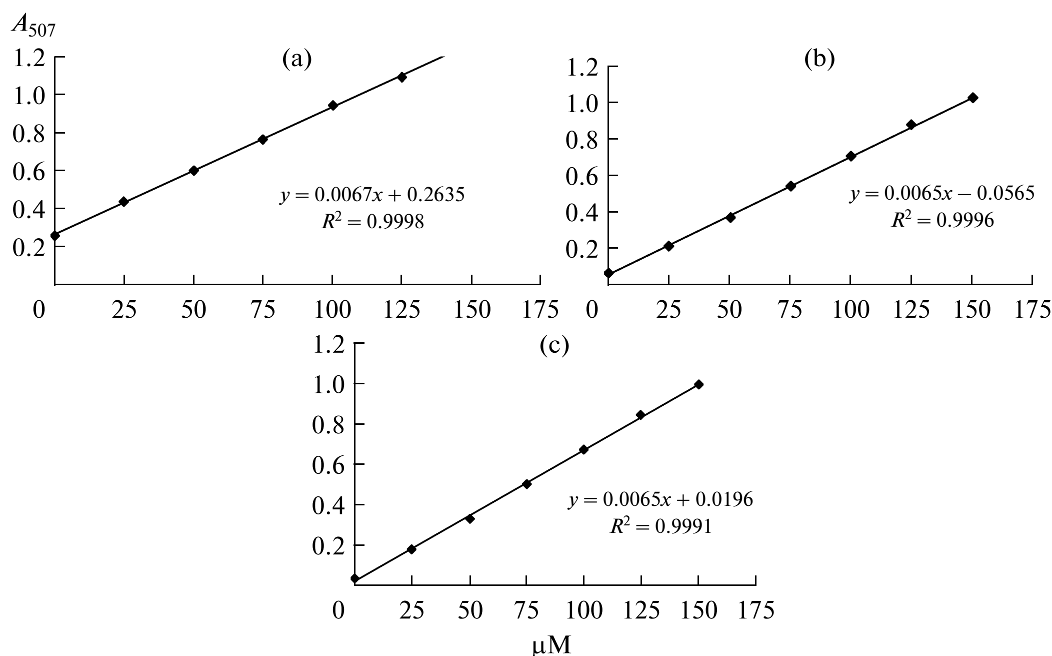

Fig. 3. Calibration curves C: A_{507nm} vs. leucine (μM) with the addition of active crude enzyme.
2.0 mM leucine was diluted to 3 sets of concentration in the range of 0–150.0 μM with 200.0 μl three concentrations dilute crude enzyme extract (diluent fold was 50, 200, or 400 respectively) and distilled water, to 1.0 ml of final volume. Dilute multiple of the active crude enzyme extract was 50 (a), 200 (b), 400 (c).

equation was $y = 0.0054x - 0.0128$ which meant that the slope was 0.0054 and the intercept was –0.0128. Figure 2 showed that the linear relationships were obtained between increasing concentrations of leucine and their color yields with the addition of three concentrations of inactive crude enzyme extract, in which a was $y = 0.0067x + 0.1609$ when the enzyme was diluted by 50 times, b was $y = 0.0065x + 0.0608$ when the enzyme was diluted by 200 times, c was $y = 0.0064x + 0.0267$ when the enzyme was diluted by 400 times (the dilute multiple of enzyme here was corresponding to the dilute multiple of enzyme–substrate reaction), the results indicated that the slopes of the three set of calibration curves were nearly the same but the intercepts were different which gradually became small when the dilute multiple of enzyme was big. Figure 3 showed that the linear relationships were obtained between increasing concentrations of leucine and their color yields with the addition of three concentrations of active crude enzyme extract, in which a was $y = 0.0067x + 0.2635$ when the enzyme was diluted by 50 times, b was $y = 0.0065x + 0.0565$ when the enzyme was diluted by 200 times, c was $y = 0.0065x + 0.0196$ when the enzyme was diluted by 400 times, the results suggested that the slopes of the three sets of calibration curves were nearly the same but the intercepts were different too. These results indicated that the addition of crude enzyme extract did greatly affect the color yield no matter whether the enzyme was active or inactive.

Absorbance after enzyme–substrate reaction and stain reaction. Table 1 showed the absorbance at 507 nm after enzyme–substrate reaction and followed stain reaction with Cd-ninhydrin, the results indicated that the absorbance was different when different enzyme concentrations were used, and it was also found that the color yield was still high even though the inactive crude enzyme extract was used as blank under the same determination condition suggesting that the crude enzyme extract itself had great influence on the color yield.

Carboxypeptidase activities. Table 2 showed the carboxypeptidase activities which were calculated according to different calibration curves, the results indicated that the calibration curves had great influence on the carboxypeptidase activity. Calibration curve A was responsible for the highest carboxypeptidase activities for all substrates because it was a pure leucine standard curve. Using such a curve, the yield color was considered to be from one source, that is, leucine was liberated from the C-terminal of Z-Xaa-Leu substrate during the enzyme-substrate reaction. However, certain unknown components in crude enzyme extract had chance to react with Cd-ninhydrin reagent and increase color development, consequently, carboxypeptidase activities could be overestimated if calibration curve A was utilized; It was a common method that an inactive enzyme was utilized to replace the active enzyme as a blank to minimize the influence of sample components which had possibility to increase the color yield under the same enzyme–substrate reaction condition and the determination condition, but the data from Table 2 indicated that the enzyme activity

Table 1. A_{507} after enzyme-substrate reaction and followed stain reaction with Cd-ninhydrin

Substrate	Diluent fold	$^{*}A_{507nm}$	$^{**}A_{507nm}$ of inactive enzyme	$^{***}\Delta A_{507nm}$
Z-Phe-Leu	1.25	0.858	0.294	0.564
		0.836	0.279	0.557
		0.851	0.309	0.542
	5	0.473	0.070	0.402
		0.487	0.071	0.416
		0.493	0.071	0.422
	10	0.225	0.020	0.205
		0.245	0.034	0.211
		0.239	0.023	0.216
Z-Trp-Leu	1.25	0.799	0.293	0.506
		0.793	0.290	0.500
		0.790	0.296	0.497
	5	0.228	0.067	0.161
		0.220	0.065	0.155
		0.222	0.069	0.153
	10	0.096	0.030	0.063
		0.093	0.036	0.060
		0.100	0.033	0.067
Z-Ala-Leu	1.25	0.451	0.263	0.188
		0.448	0.266	0.182
		0.445	0.260	0.185
	5	0.109	0.063	0.046
		0.113	0.061	0.050
		0.114	0.066	0.051
	10	0.056	0.029	0.026
		0.054	0.030	0.024
		0.063	0.030	0.033

* A_{507nm}: Absorbance at 507 nm from solution of active crude enzyme and substrate, colored by Cd-ninhydrin reagent.
** A_{507nm} of inactive enzyme: Absorbance at 507 nm from solution of inactive crude enzyme and substrate, colored by Cd-ninhydrin reagent.
*** $\Delta A_{507nm} = {}^{1}A_{507nm} - {}^{2}A_{507nm}$.

calculated with this method was still higher than the enzyme activity calculated with calibration curve B and calibration curve C suggesting that this method could not eliminate the influence effectively; There was no significant difference between the enzyme activity calculated with calibration curve B and the enzyme activity calculated with calibration curve C when proper dilute multiple of enzyme was applied, suggesting that the standard addition method was needed to eliminate the influence of background of crude enzyme extract on the activity determination.

Effect of enzyme dilute folds on absorbance and carboxypeptidase activity. The determination of enzyme activity was usually affected by dilute folds, as displayed in Tables 1 and 2. As could be seen from the results of substrate Z-Phe-Leu, the absorbance value was high when the enzyme was diluted 1.25 times, while the enzyme activity was so low, but there was no

Table 2. Carboxypeptidase activities* in *Actinomucor elegance* crude enzyme extract calculated by calibration curve A, calibration curve B and calibration curve C

Substrate	Diluent fold	Calibration curve A	**Calibration curve A (ΔA_{507nm})	Calibration curve B	Calibration curve C
	1.25	6.65 ± 0.09	4.62 ± 0.09	4.28 ± 0.07	3.61 ± 0.05
Z-Phe-Leu	5	15.34 ± 0.32	13.18 ± 0.32	10.86 ± 0.26	10.97 ± 0.26
	10	15.89 ± 0.34	13.86 ± 0.34	11.35 ± 0.29	10.54 ± 0.29
	1.25	6.22 ± 0.04	4.20 ± 0.03	3.94 ± 0.03	3.30 ± 0.03
Z-Trp-Leu	5	7.29 ± 0.12	5.13 ± 0.13	4.17 ± 0.11	4.28 ± 0.11
	10	6.74 ± 0.22	4.70 ± 0.22	4.17 ± 0.18	3.93 ± 0.18
	1.25	3.56 ± 0.03	1.53 ± 0.02	1.79 ± 0.02	1.15 ± 0.02
Z-Ala-Leu	5	3.85 ± 0.08	1.91 ± 0.09	1.31 ± 0.06	1.42 ± 0.06
	10	4.35 ± 0.29	2.50 ± 0.29	2.19 ± 0.24	1.95 ± 0.25

* Caboxypeptidase activities expressed as μmol of leucine h^{-1} ml^{-1} of the *A. elegans* extract.

** Carboxypeptidase activities calculated by calibration curve A and ΔA_{507nm}; $\Delta A_{507nm} = A_{507nm}$ after enzymatic reaction between active crude enzyme and substrate – A_{507nm} after enzymatic reaction between inactive crude enzyme and substrate.

significant difference between the enzyme activity monitored using 5 times dilution enzyme solution and the enzyme activity monitored using 10 times dilution enzyme solution, because both of the dilute folds were all suitable for the enzyme-substrate reaction of this substrate. The carboxypeptidase activity for substrate Z-Trp-Leu and Z-Ala-Leu further obtained from Table 2 conformed that there were at least 2 times of dilute folds were suitable for the determination of enzyme activity which has already been applied on the peptidase activity determination [11].

The results obtained from present study indicated that the standard addition method was necessary for the determination of carboxypeptidase in *Actinomucor elegans* bran koji, and maybe suitable for the determination of other enzyme activity in crude extract without purification to meet the demand of precision and objectivity.

ACKNOWLEDGEMENTS

This Project was supported by the National Natural Science Foundation of China (Grant No. 30770056).

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