An analytical method for measuring α-amylase activity in starch-containing foods

Title Page

Title

An Analytical Method for Measuring α-Amylase Activity in Starch-Containing Foods

Authors

Kazuo Koyama[a,b,*], Takashi Hirao[a], Akira Toriba[b], and Kazuichi Hayakawa[b]

Affiliations

a) Somatech Center, House Foods Corporation, 1-4 Takanodai, Yotsukaido-shi, Chiba 284-0033, Japan

b) Graduate School of Natural Science and Technology, Institute of Medical Pharmaceutical and Health Science, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

Corresponding author

Name: Kazuo Koyama

E-mail: k-koyama@housefoods.co.jp

Telephone: +81 43 237 5211

Fax: +81 43 237 2914

Text

Abstract

The quality of starch-containing foods may be significantly impaired by contamination with very small amounts of α-amylase, which can enzymatically hydrolyze the starch and cause viscosity loss. Thus, for quality control, it is necessary to have an analytical method that can measure low amylase activity. We developed a sensitive analytical method for measuring the activity of α-amylase (from *Bacillus subtilis*) in starch-containing foods. The method consists of six steps: 1) crude extraction of α-amylase by centrifugation and filtration, 2) α-amylase purification by desalting and anion-exchange chromatography, 3) reaction of the purified amylase with boron-dipyrromethene (BODIPY)-labeled substrate, which releases a fluorescent fragment upon digestion of the substrate, thus avoiding interference from starch derivatives in the sample, 4) stopping the reaction with acetonitrile, 5) reversed-phase solid-phase extraction (SPE) of the fluorescent substrate to remove contaminating dye and impurities, and 6) separation and measurement of BODIPY fluorescence by HPLC. The proposed method could quantify α-amylase activities as low as 10 mU/ml, which is enough to reduce the viscosity of starch-containing foods.

Key Words

α-Amylase; Starch-containing foods; Fluorescent substrate; Purification; High-performance liquid chromatography

Introduction

Honey, raw vegetables, spices, grains and fermented food are potential sources of amylase. These foods also are used as materials in thickening sauces that contain wheat flour or corn starch, such as canned sauces or soups and retort-packed stews or curries. The increase in viscosity of starch-containing foods is largely due to the starch gelatinization during heating in the factory, but small amounts of amylase can hydrolyze the starch, leading to viscosity loss and degradation of food quality. Addition of honey was found to cause a loss of viscosity in a waxy maize suspension (Babacan *et al*., 2002). In the preparation of starch-containing foods, raw materials that contain amylase are heated to denature the enzyme before they are mixed with starch, but the denaturation is not always complete. When the viscosity of the starch-containing foods is below the acceptable range, the food must be analyzed for residual amylase activity.

Contamination with amylase can also occur at home if a consumer incorrectly cooked starch-containing foods, such as instant powdered soup, canned white sauce, and retort curry sauce. Possible sources of amylase contamination include saliva left on a tasting spoon (containing human amylase), honey used for sweetener, added vegetables and spices, and dishwasher detergent remaining on dishes (which might contain bacterial amylase). Saliva contamination was found to reduce the viscosity of a maize starch-thickened drink used in the clinical management of dysphagia (Hanson *et al*., 2012). If the viscosity of sauce cooked at home was lower than that previously experienced, a consumer would complain and send cooked food back to the manufacturer. The manufacturer would analyze the sample in order to explain the reason of viscosity loss to the consumer.

Conventional methods for measuring amylase activity include monitoring the degradation of chromogenic substrates (Filova *et al*., 1992) such as blue starch (Miwa *et al*., 1982), *β*-Limit dextrin (Kruger *et al*., 1972), or *p*-nitrophenyl derivatives of oligosaccharides (Wallenfels *et al*., 1982; Moneghini *et al*., 1989; Tokutake *et al*., 1992). These methods, which are commonly used for biological samples (*ca*. serum, plasma, urine) or sprouted grain (*ca*. wheat, barley, malt), have insufficient sensitivity (limits of quantification >1 U/ml) to detect trace amounts of amylase contaminating starch-containing foods during cooking.

Another method uses a fluorescent substrate labeled with boron-dipyrromethene (BODIPY) (Molecular Probes, 2006; Haugland *et al*., 1998; Zhou *et al*., 1998). This method, which can measure 2 mU/ml of amylase activity in blood samples, is sufficiently sensitive, but it is not applicable for food analysis because foods typically contain many interfering substances.

We propose a new method for quantifying trace amounts of α-amylase activity contaminating starch-containing foods by using a *Bacillus subtilis* α-amylase model. To improve sensitivity, α-amylase is purified before reacting with the BODIPY-labeled substrate to avoid competitive inhibition by starch derivatives in the food sample.

Materials and methods

Reagents and food materials

α-Amylase from *Bacillus subtilis*, starch from wheat, NaCl, tris(hydroxymethyl)aminomethane, HCl, NaOH, ammonium acetate, acetonitrile, and iodine solution were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-Morpholinopropanesulfonic acid (MOPS) was purchased from Dojindo Laboratories (Kumamoto, Japan). As the fluorescent substrate, the dye quenching starch (DQ starch) labeled with BODIPY FL was obtained from Life Technologies Corp. (NY, USA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Japan (Tokyo). Curry roux was purchased from a local market.

α-Amylase standard

The α-amylase solutions were prepared for spiking experiments and constructing a calibration curve. The α-amylase solution (0.75－25 U/ml) used for spiking experiment was prepared by dissolving the α-amylase (*Bacillus subtilis*, EC 3.2.1.1, 111 U/mg solid) with 100 mM MOPS buffer, pH 6.9. The α-amylase solution used for calibration curve (0.75－12.5 mU/ml) was prepared by dissolving the α-amylase with 20 mM tris-HCl buffer containing 0.2 M NaCl, pH 8.0.

Food models

Two types of food models were prepared to evaluate our method. A starch suspension model was prepared by mixing an aqueous solution of 3% starch and 1.1% NaCl, and then boiling for 5 min to thicken the slurry by gelatinization. A curry sauce model was prepared by boiling an aqueous solution of 10% commercial curry roux (including starch and NaCl) for 5 min. The starch suspension and curry sauce were sterilized at 121ºC for 15 min and stored in plastic tubes with screw-caps at room temperature until use.

Time course study of viscosity loss by α-amylase

The starch suspensions were re-gelatinized by placing the plastic tubes in boiling water for 5 min and then allowing them to cool in a 40ºC water bath for 30 min. Then, 100 μl of 0.75, 2.5, 7.5 U/ml α-amylase solutions and 100 μl of 25% (w/v) BSA solution were added to 25 ml of starch suspensions to obtain test samples containing 3, 10, and 30 mU/ml of α-amylase. Viscosity was measured with a viscometer DVL-B2 equipped with HM-2 rotor (Toki Sangyo Co., Ltd., Tokyo, Japan) at 60 rpm.

Test samples for α-amylase recovery study

The starch suspension and curry sauce models were re-gelatinized as described above. Then, 100 μl of 0.75, 2.5, 7.5, 25 U/ml α-amylase solutions and 100 μl of 25% (w/v) BSA solution were added to 25 ml of the food models to obtain test samples containing 3, 10, 30, and 100 mU/ml of α-amylase and incubated at 40ºC water bath for 30 min and applied for amylase assay.

α-Amylase assay

Outlines of the proposed method and the conventional method are shown in Table 1.

Proposed method

(i) **Crude extraction**; To remove insoluble starch and contaminants in the test samples, 25 ml of the incubated starch suspensions and the curry sauces containing 10, 30, and 100 mU/ml of α-amylase were centrifuged at 20,000 × g for 10 min. The supernatant was filtered

through No. 5B filter paper and named crude extract.

(ii) **α-Amylase purification**; Pre-purification steps were conducted to exclude competitive inhibitors by starch derivatives from the crude extract. The crude extract (1.0 ml) was desalted by applying it to a PD MiniTrap G-25 column (3.5 ml gel, GE Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with 20 mM tris-HCl, pH 8.0 (buffer A). The desalted amylase was eluted with 1.5 ml of buffer A and loaded on a DEAE-Toyopearl 650M (anion-exchange) column (1.5 ml gel, Tosoh Corp., Tokyo, Japan) equilibrated with buffer A. After the column was washed with 6.0 ml of buffer A, the α-amylase was eluted using 4.0 ml of 0.2 M NaCl in buffer A (buffer B). The α-amylase-containing pre-purified fraction was stabilized by addition of 0.1% BSA.

(iii) **α-Amylase-fluorescent substrate reaction**; Fifty microliters of the purified α-amylase solution was added to 50 μl of 200 μg/ml BODIPY-labeled DQ starch dissolved in 100 mM MOPS buffer, pH 6.9 and the mixture was incubated for 30 min at room temperature in the dark. BODIPY-labeled DQ starch is a quenched starch substrate that yields fluorescent fragments on amylase digestion. In case of high α-amylase activity (100 mU/ml), the sample was diluted two-fold with buffer B and applied for the reaction. A calibration curve was created by using 50 μl of blank sample and 0.75, 2.5, 7.5, 12.5 mU/ml α-amylase solutions (corresponding to test samples containing 3, 10, 30, 100 mU/ml).

(iv) **Stopping enzymatic reaction**; One hundred microliters of acetonitrile was immediately added to the reaction mixture and mixed to inactivate the α-amylase. The mixture was centrifuged at 13,800 × g for 10 min.

(v) **Purification of fragment of fluorescent substrate**; A post-purification step was conducted to remove dyes and impurities derived from the food samples. The centrifuged supernatant was loaded on a C18 SPE cartridge (100 mg, Bond Elute, Agilent Technologies Inc., CA, USA) and eluted with the mixture of 20 mM ammonium acetate aqueous / acetonitrile (1/1, v/v). The eluent was filled up to 1.0 ml and was filtered with a 0.20 μm membrane.

(vi) **HPLC Measurement**; The fluorescence of the test solution was measured by HPLC. An Agilent 1100 Series HPLC system consisting of a quaternary pump, a vacuum degasser,

an auto injector, a column oven, and a fluorescence detector was used. The fragment of fluorescent substrate was separated on an L-column2 C18 (2.1 mm × 150 mm, 3 μm, CERI, Saitama, Japan) with binary gradient. Mobile phases A and B were 20 mM ammonium acetate aqueous solution and acetonitrile, respectively. The gradient was as follows; 10－25% B from 0 to 7 min, 25－65% B from 7 to 17 min, 65－95% B from 17 to 20 min, 95－10% B from 20 to 29 min (a total run time of 40 min). The flow rate was 0.2 ml/min and the column temperature was kept at 40℃. The fluorescence wavelength of the detector was set at Ex / Em = 502 / 512 nm.

LC-MS/MS analysis of fragment of fluorescent substrate

The structure of the fragments of the fluorescent substrate was analyzed by LC-MS/MS, consisting of an Acquity UPLC and a Quattro Premier XE Mass Spectrometer system (Waters, MA, USA). The fragment of fluorescent substrate was separated on a ZIC-HILIC column (2.1 mm×100 mm, 5 μm, Merck SeQuant AB, Umeå, Sweden) with binary gradient. The mobile phases and flow rate and the column temperature were as described in step (vi). The gradient was 90－60% B from 0 to 17 min. MS detection was performed in positive ESI mode with a capillary voltage of 3.0 kV, a cone voltage of 10 V, the source block temperature set to 130℃ and desolvation temperature set to 450℃. Nitrogen gas was used as both the desolvation and cone gas, which were 800 and 50 l/min. A product ion scan of precursor ions was obtained with a collision energy of 30 eV. Mas range was set to 150－2000 for the precursor ion scan and to 50－1700 for the product ion scan. Mixtures of 10 μl of 10 U/ml α-amylase and 1 mg/ml fluorescent substrate (DQ starch) were incubated for 30 min, mixed with 80 μl of acetonitrile and filtered. An aliquot of the filtrate was then injected into the column.

Conventional method

A conventional amylase assay was conducted with an EnzChek Ultra Amylase Assay Kit (Life Technologies Corp.) according to the instructions of the manufacturer (Molecular Probes, 2006). As described previously, the crude extract obtained by step (i) was diluted

four-fold with buffer B and applied for substrate reaction as described in step (iii). The fluorescence intensity of the reactant was measured with a Synergy HT microplate reader (BioTek Instruments Inc., VT, USA), at Ex / Em = 485 ± 10 nm / 528 ± 10 nm.

Efficiency of α-amylase purification step

Separation of the spiked α-amylase and the soluble starch derivatives in the food models at the pre-purification step was checked as follows. A starch suspension spiked with 100 mU/ml α-amylase was extracted as described in step (i). All fractions of the PD MiniTrap G-25 column and the DEAE-Toyopearl 650M column were collected. For α-amylase analysis, 50 μl of each fraction was incubated with 50 μl of fluorescent substrate (DQ starch) for 30 min. The fluorescence was immediately measured with a microplate reader. Chloride ion concentration was measured with a salt analyzer STA-210 (DKK-TOA Corp., Tokyo, Japan). For starch analysis, 50 μl of each fraction and 50 μl of 2 mM iodine solution were mixed and the absorbance of the mixture was measured using a microplate reader at 620 nm.

Results and discussion

Time course of viscosity loss by α-amylase

Figure 1 shows the time course of the viscosity loss at 40°C observed in starch suspensions spiked with 3, 10, and 30 mU/ml of *Bacillus subtilis* α-amylase. The incubation temperature, 40°C, was adopted so as to simulate a viscosity loss during the cooling step after cooking. The viscosity was rapidly reduced, especially in the samples containing more than 10 mU/ml α-amylase and was almost lost within 20 min. This result indicated that a new analytical method was required to detect trace amounts of α-amylase activity (i.e., in the range of 3 − 30 mU/ml) in order to investigate the cause of the viscosity loss of starch-containing foods.

Efficiency of pre-purification step

In the starch suspension and curry sauce, the crude extraction was performed to remove

insoluble starch. To avoid the competitive inhibition caused by soluble starch derivatives included in the food samples, the combination of gel-filtration chromatography and anion-exchange chromatography (AEC) was introduced in the proposed method as α-amylase pre-purification steps prior to the α-amylase-fluorescent substrate reaction step.

In conventional methods for purifying amylase, amylase is retained on an anion-exchange resin with alkaline buffer (buffer A) and then is eluted with the same buffer containing 1.0 M NaCl (buffer B) (Mitsuiki *et al*., 2005; Babacan *et al.*, 2005). However, because many foods contain salts, the samples must be desalted before AEC. In this study, the crude extract was loaded on the PD MiniTrap G-25 column. Figure 2 shows that the salt in test sample was separated from α-amylase containing soluble starch derivatives. The desalted sample was then loaded on the DEAE-Toyopearl 650M anion-exchange column (Figure 3). α-Amylase was clearly separated from the soluble starch in the desalted extract. The anion-exchange resin held the α-amylase while the soluble starch was removed with buffer A, then the α-amylase was eluted with buffer B containing 0.2 M NaCl. AEC using buffer B with a low salt concentration could lessen the elution of the dye derived from curry sauce. Moreover, α-amylase reacted better with the fluorescent substrate in 0.2 M NaCl than in 1.0 M NaCl. If the crude extract was directly loaded on the DEAE-Toyopearl 650M anion-exchange column, α-amylase could not be separated from soluble starch derivatives (Figure 3, triangular points).

Post-purification and HPLC detection of fluorescent substrate

In the proposed method, after the α-amylase-fluorescent substrate reaction, the post-purification steps consisting of reversed-phase SPE were performed to remove dye and impurities in food samples. The fragments of fluorescent substrate were measured with reversed-phase HPLC with a fluorescence detector to avoid the above influences. Figure 4 shows that the peaks accompanying by α-amylase activity detected in starch suspension and curry sauce. The broad peak at a retention time of ca. 17 min was composed of multiple fragments of fluorescent substrate. The fragments were not retained on the C18 column when the mobile phase used a higher percentage of acetonitrile, used methanol, or when the mobile

phase did not contain ammonium acetate. This result suggested that the fragments were hydrophilic and probably anionic.

The broad peak fraction was analyzed by LC-MS/MS using the hydrophilic interaction chromatography (HILIC) mode. The precursor ions of the fragments of the fluorescent substrate were detected as $[M+H]^+$ at m/z 853, 1015, 1177, 1339, 1501, and 1664 (Fig. 5), indicating a polysaccharide-specific m/z spacing patterns (162 mass units) (Sarver *et al*., 2012). Figure 6 shows the product ion mass spectrum of m/z 1177 at RT 10.5 min of Figure 5. The spectrum shows the product ions minus one BODIPY group of 219 mass (Jang *et al*., 2006) and at least three glucose residues of 162 mass units. The same spectra were obtained from the other precursor ions. These results indicate that the area of the broad peak of Figure 4, containing several lengths of the BODIPY-labeled hydrolyzed polysaccharide, is appropriate for the quantification of α-amylase activity.

Figure 7 shows the dependence of fluorescent intensity on α-amylase activity of standard solutions and food models of (a) starch suspension and (b) curry sauce. The calibration curves from the peak area increased as the square of the α-amylase activity, because there was a small amount of interference with the rate of the fluorescent substrate reaction. However, the calibration curve from food models made it possible to analyze actual unknown food samples. Although the signals from the food models were smaller than the ones from the standard solutions, the differences in the signal intensities were small enough to not appreciably affect measurement of α-amylase activity.

Recovery test

To verify the applicability of the methods to food samples, the recovery tests were performed with the starch suspension and the curry sauce spiked with 10, 30, and 100 mU/ml α-amylase (Figure 8). The conventional methods using the EnzChek Ultra Amylase Assay Kit could not detect the amylase activity in the food models. The proposed method could quantify the range of 10－100 mU/ml of α-amylase activities with a satisfactory recovery rate from 87 to 101% in the starch suspensions and from 74 to 104% in the curry sauce.

The conventional method could not detect α-amylase activities lower than 100 mU/ml in

food models, but could detect 2 mU/ml of standard solution according to product data for the EnzCheck assay.

The method without pre-purification, in which the crude extraction and post-purification steps were added to the conventional method (Table 1, middle column), could detect α-amylase activity in food models as low as 30 mU/ml (Figure 6, hacthed bars). The improvement of the sensitivity is due to the removal of insoluble starch and dye. But the recovery rates were proportionally decreased with the amylase concentration. This result indicates that the crude extraction and post-purification steps were insufficient to reduce the competitive inhibition by the soluble starch derivatives contained in the crude extract (white colored with cloudy appearance). To obtain higher sensitivity, the proposed method should include the crude extraction, α-amylase pre-purification, fluorescent substrate reaction, and purification of fragment of fluorescent substrate steps.

In the proposed method, the α-amylase pre-purification steps consisting of desalting and anion-exchange chromatography (AEC) dramatically improved the recovery rates of α-amylase in starch suspensions. This result clearly shows that the pre-purification steps of the soluble starch from the crude extract, was essential for the elimination of competitive inhibition. The proposed method for curry sauce also gave a satisfactory recovery rate. This result indicated that the post-purification steps consisting of reversed-phase SPE and HPLC were useful to remove the dyes and the impurities in the curry sauce as shown in Figure 4. The proposed method could quantify 10 mU/ml of α-amylase activity which can cause the starch suspension to lose viscosity within 20 min (Figure 1). The limit of detection of the proposed method is 3mU/ml of α-amylase activity which reduced the viscosity of the starch suspension, because the calibration curves (Figure 7) were approximately linear in the range corresponding to the activities of the test samples (3－100 mU/ml).

Conclusion

We developed a novel analytical method to quantify α-amylase activities as low as 10 mU/ml, which can cause viscosity loss in starch-containing foods. The method is based on monitoring the fluorescent substrate digested by α-amylase combined with steps to avoid

interferences caused by starch, dye, and impurities in food samples. The desalting and anion exchange chromatography steps removed the competitive inhibition caused by starch in the food sample. The reversed-phase SPE and HPLC steps made it possible to measure the fragment of substrate without interference from dye and impurities in food samples. This is the first report that α-amylase can be determined at trace levels that can cause a loss of viscosity in starch-containing foods.

References

Babacan S, Pivarnik LF and Rand AG. Honey amylase activity and food starch degradation. Journal of Food Science 2002; **67**: 1625-1630.

Babacan S and Rand AG. Purification of amylase from Honey. Journal of Food Science 2005; **70**: C413－C418.

Hanson B, O'Leary MT and Smith CH. The effect of saliva on the viscosity of thickened drinks. Dyshpagia 2012; **27**: 10-19.

Filova V, Valentova O, Daussant J and Kas J. Some methods for isolation and assays of enzymes occurring in cereals and legumes. Food Reviews International 1992; **8**: 559－572.

Haugland RP and Zhou M. Dye labeled polymers as reagents for measuring polymer degradation. US Patent 5719031, 1998.

Jang HG, Park M, Wishnok JS, Tannebaum SR and Wogan GN. Hydroxy-specific fluorescence labeling of ABP-deoxyguanosine, PhIP-deoxyguanosine, and AFB1-formamidopyrimidine with BODIPY-FL. Analytical Biochemistry 2006; **359**: 151－160.

Kruger JE and Marchylo B. The use of reduced β-limit dextrin as substrate in an automated amylase assay. Cereal Chemistry 1972; **49**: 453－458.

Mitsuiki S, Utsunomiya H, Nakama Y, Sakai M, Mukae K, Moriyama Y, Goto M and Furukawa K. Purification and Characterization of Maltotriose-producing Amylases from an Alkaliphilic *Nocardiopsis* sp. TOA-1. Journal of Applied Glycoscience 2005; **52**: 95－99.

Miwa J. Medico-legal studies on the human saliva (Part 3). Nihon University Dental Journal

1982; **56**: 413－419.

Molecular Probes. EnzChek Ultra Amylase Assay Kit (E33651), 2006. http://www.invitrogen.jp/products/pdf/mp33651.pdf

Moneghini M, Maurich V, Sciortino T, Solinas D, Franchi G and Lencioni E. A High-performance liquid chromatgraphic method for the determination of amylase (*p*-nitrophenyl-α-maltosidase) activity. Journal of Pharmaceutical & Biomedical Analysis 1989; **7**: 513－517.

Sarver SA, Keithley RB, Essaka DC, Tanaka H, Yoshimura Y, Palcic MM, Hindsgaul O, Dovichi NJ. Preparation and electrophoretic separation of Bodipy-Fl-labeled glycosphingolipids. Journal of Chromatogr A 2012; **1229**: 268-273.

Tokutake S, Kotani K, Saito K and Yamaji N. Sytheses of 2-chloro-4-nitrophenyl β-D-maltopentaosides with bulky modification and their application to the differential assay of human α-amylases. Chemical & Pharmaceutical Bulletin 1992; **40**: 2531－2536.

Wallenfels K, Laule G and Meltzer B. Action pattern of human pancreatic and salivary α-amylase on 1,4-α-*D*-nitrophenylmaltooligosaccharides. Journal of Clinical Chemistry and Clinical Biochemistry 1982; **20**: 581－586.

Zhou M, Zhang C, Upson RH and Haugland RP. Two fluorometric approches to the measurement of dextranase activity. Analytical Biochemistry 1998; **260**: 257－259.

Table 1. Outline of methods for the determination of α-amylase activity.

Step	Proposed method	Method without pre-purification	Conventional method
Crude extraction	+	+	−
α-Amylase purification (Pre-purification)	+	−	−
α-Amylase - fluorescent substrate reaction	+	+	+
Stopping enzymatic reaction	+	+	−
Purification of fragment of fluorescent substrate (Post-purification)	+	+	−
Measurement	HPLC	HPLC	Microplate reader

−, without; +, with

Figure 1

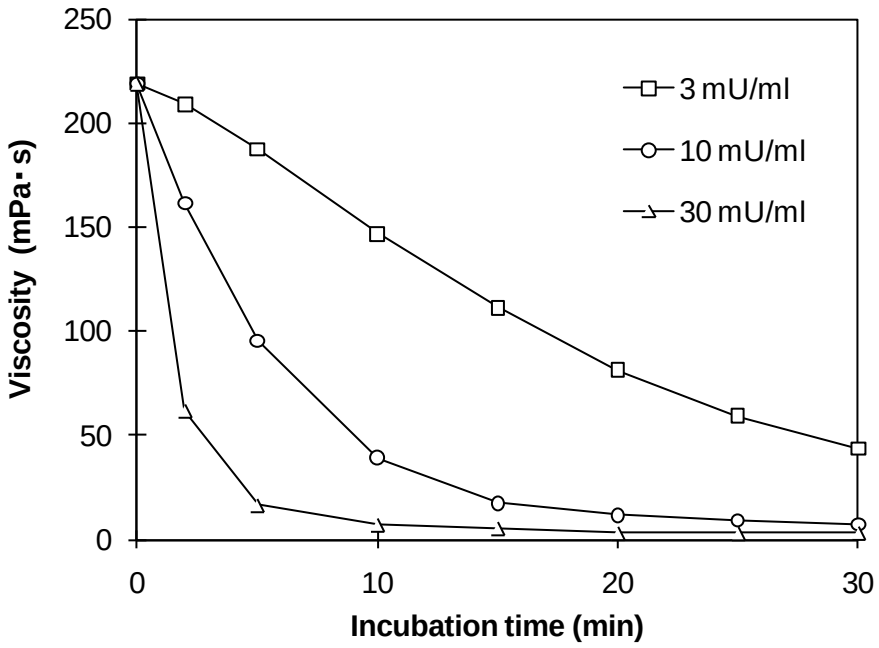

Figure 1. Time course of viscosity loss of starch suspension by α-amylase. The starch suspensions were spiked with 3, 10, 30 mU/ml α-amylase and incubated for 30 min at 40℃.

Figure 2

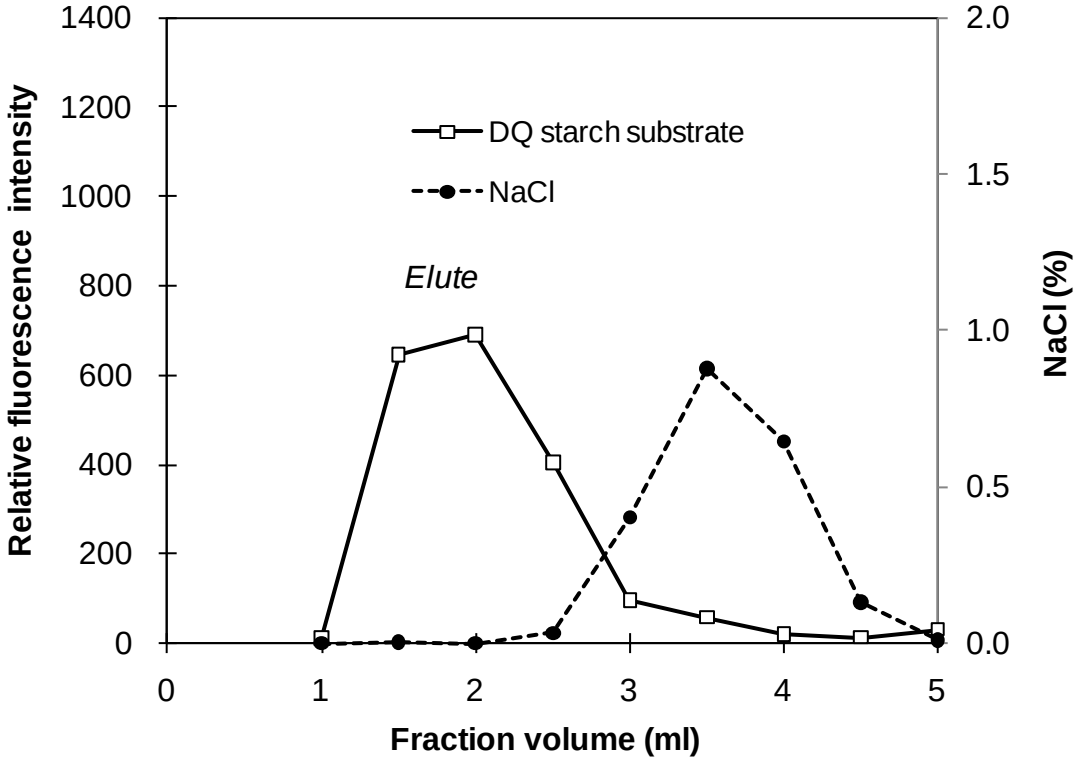

Figure 2. Gel-filtration chromatogram showing separation of α-amylase and salt from a crude extract. The crude extract of 1.0 ml was applied to a PD MiniTrap G-25 column. The desalted eluent with 1.5 ml of 20 mM tris-HCl buffer, pH 8.0. The solid curve shows fluorescence intensity from reaction of α-amylase activity and DQ starch substrate, as measured by microplate reader. The dashed curve shows chloride ion concentration measured by salt analyzer.

Figure 3

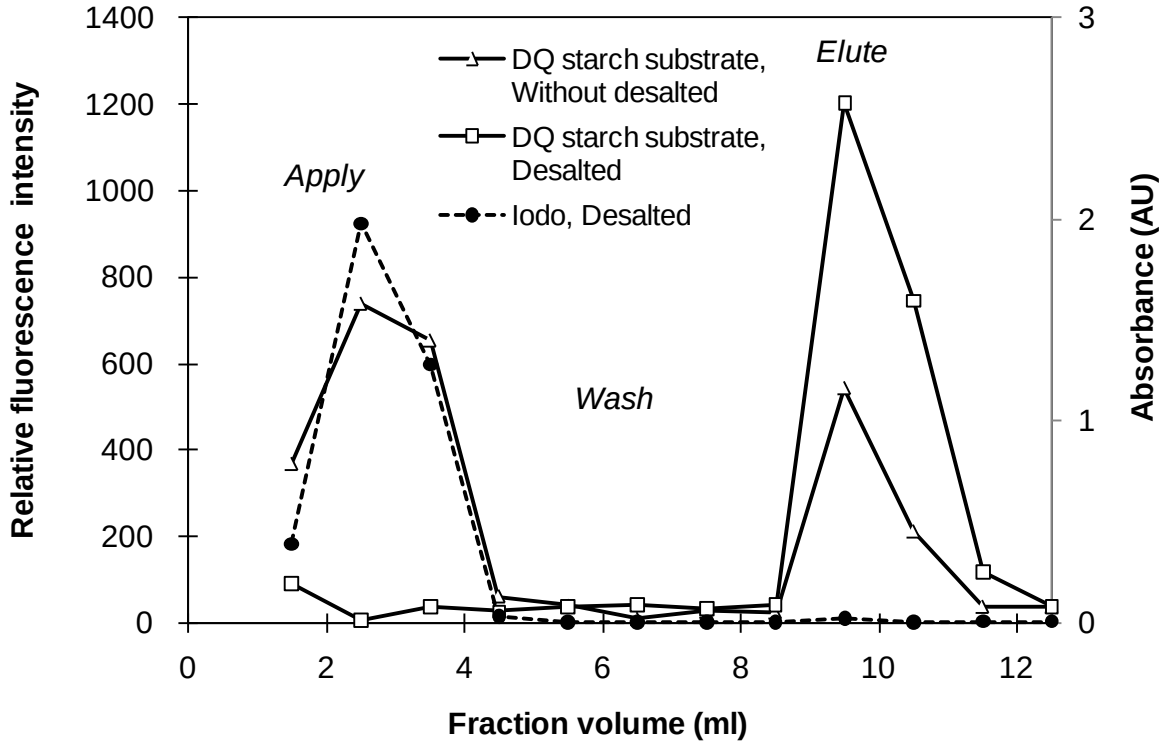

Figure 3. Anion-exchange chromatogram showing separation of α-amylase and starch from a desalted sample. The desalted α-amylase solution of 1.5 ml was applied to a DEAE-Toyopearl 650M column. The column was washed with 6 ml of 20 mM tris-HCl buffer, pH 8.0 and eluted with 4 ml of 0.2M NaCl in the same buffer. The solid curve shows fluorescence intensity from reaction of α-amylase activity and DQ starch substrate, as measured by microplate reader. The dashed curve shows absorbance from iodine-starch reaction measured by microplate reader.

Figure 4

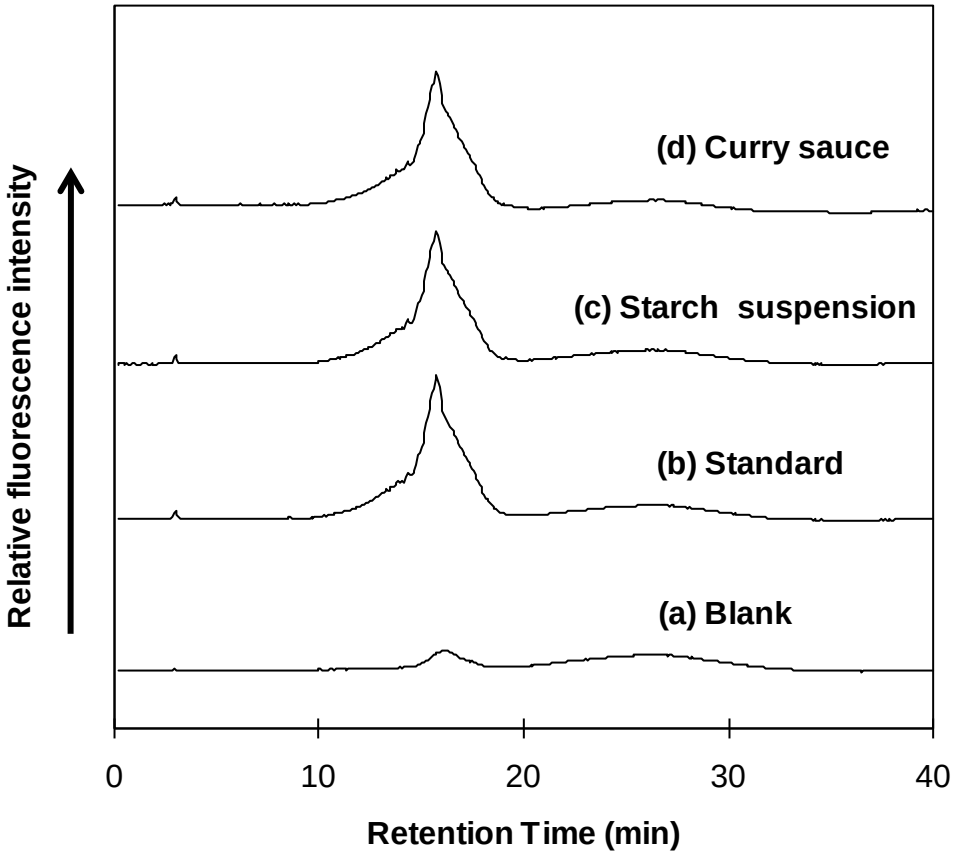

Figure 4. HPLC profiles of α-amylase activity. Solid lines show chromatograms of the fragments of fluorescent substrate (RT 17 min). (a) Blank sample from inactive α-amylase, (b) Standard from 50 mU/ml α-amylase, (c) starch suspension, (d) curry sauce. (c) and (d) were spiked with 100 mU/ml α-amylase and were diluted two fold.

Figure 5

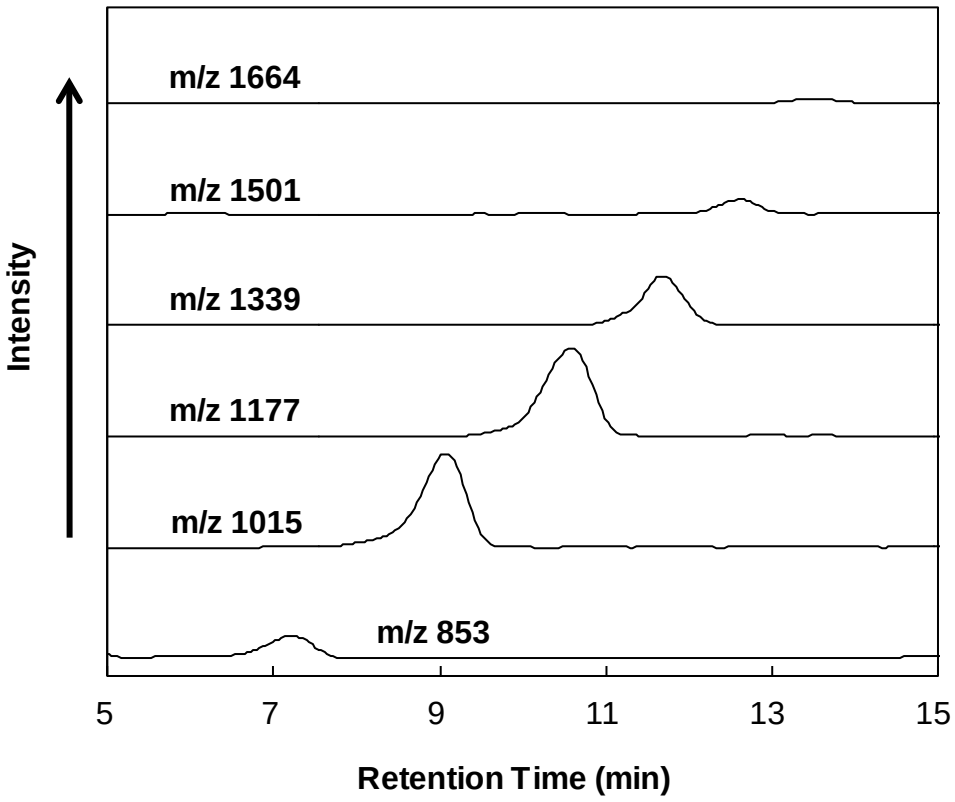

Figure 5. LC-MS scan chromatograms of fragments of fluorescent substrates from 10 U/ml α-amylase standard. LC-MS was performed in the hydrophilic interaction chromatography mode.

Figure 6

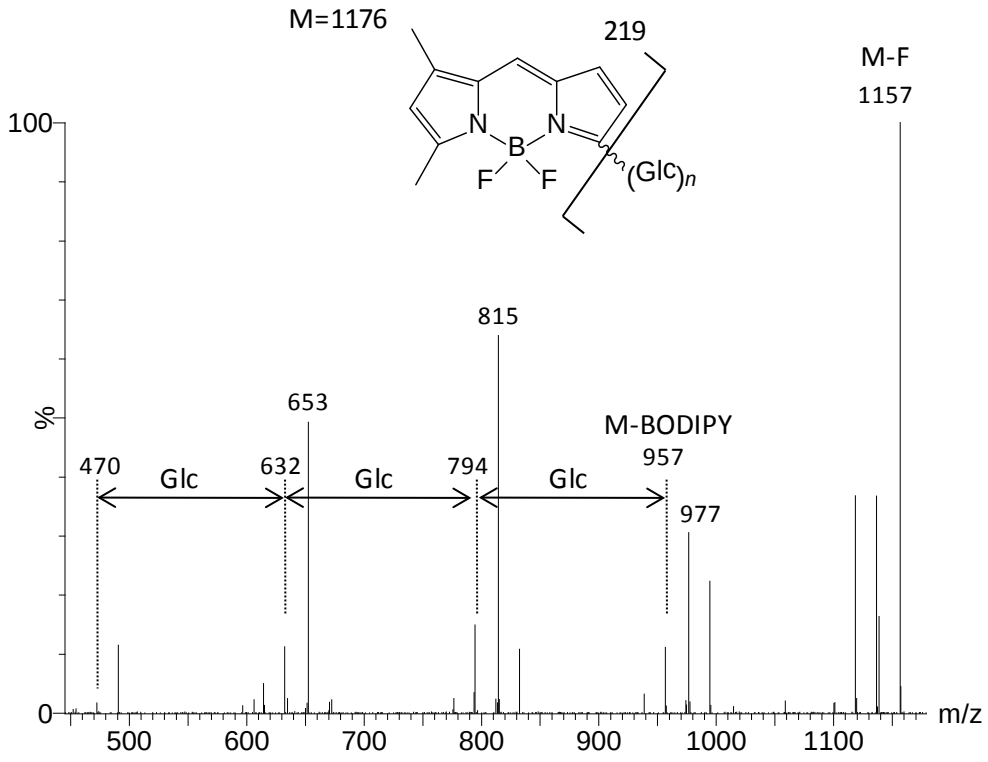

Figure 6. Ion mass spectrum of a representative fragment of fluorescent substrate (the peak at m/z 1177 $[M+H]^+$ at RT 10.5 min in Figure 5).

Figure 7

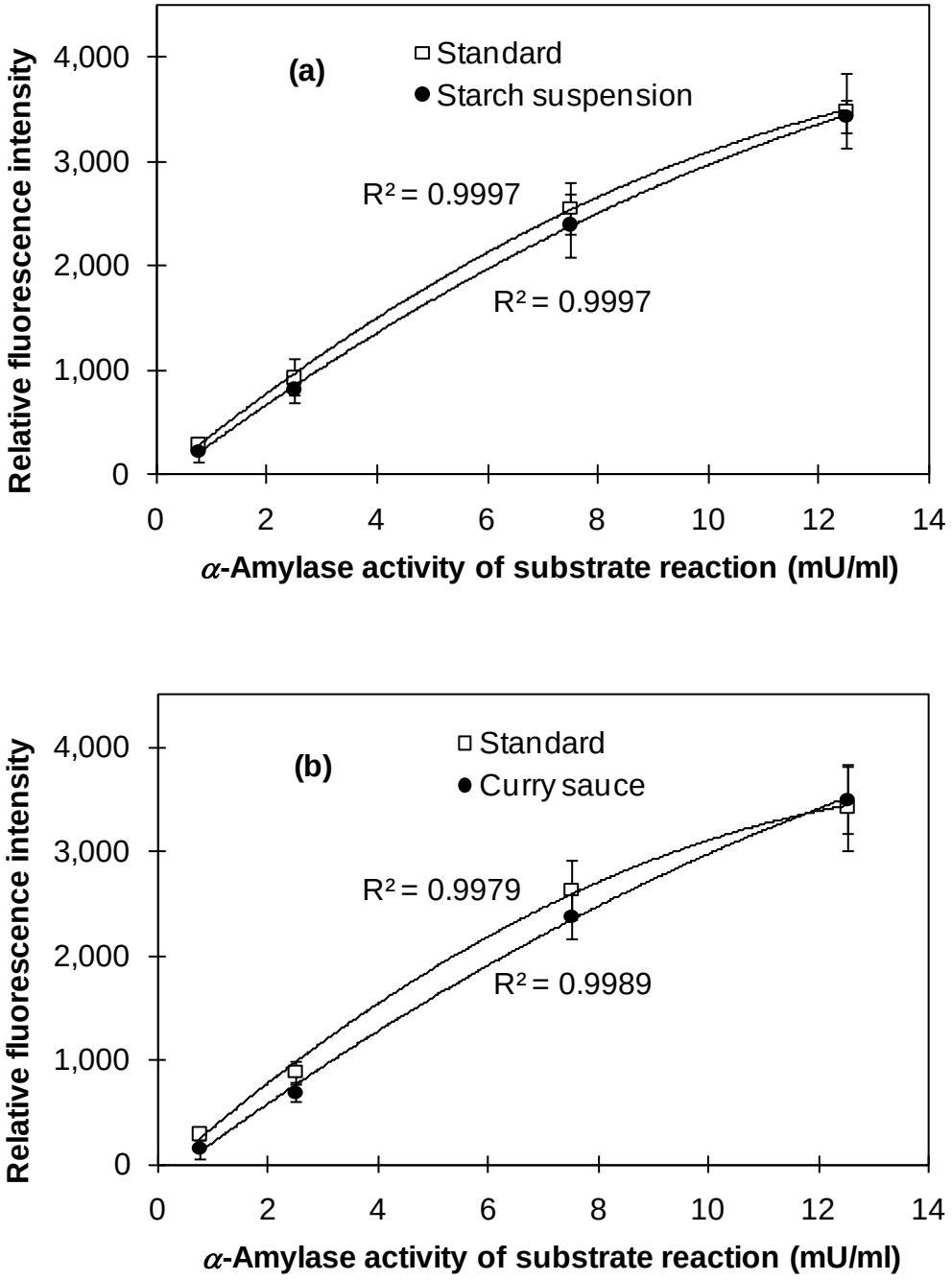

Figure 7. Fluorescent intensities expressed as the peak areas in the HPLC chromatograms. The proposed method was performed in order to recover the α-amylase from (a) starch suspension and (b) curry sauce. The standards are 0.75, 2.5, 7.5, 12.5 mU/ml, corresponding to the test samples containing 3, 10, 30, 100 mU/ml. Values shown are means ± SD obtained from five experiments.

Figure 8

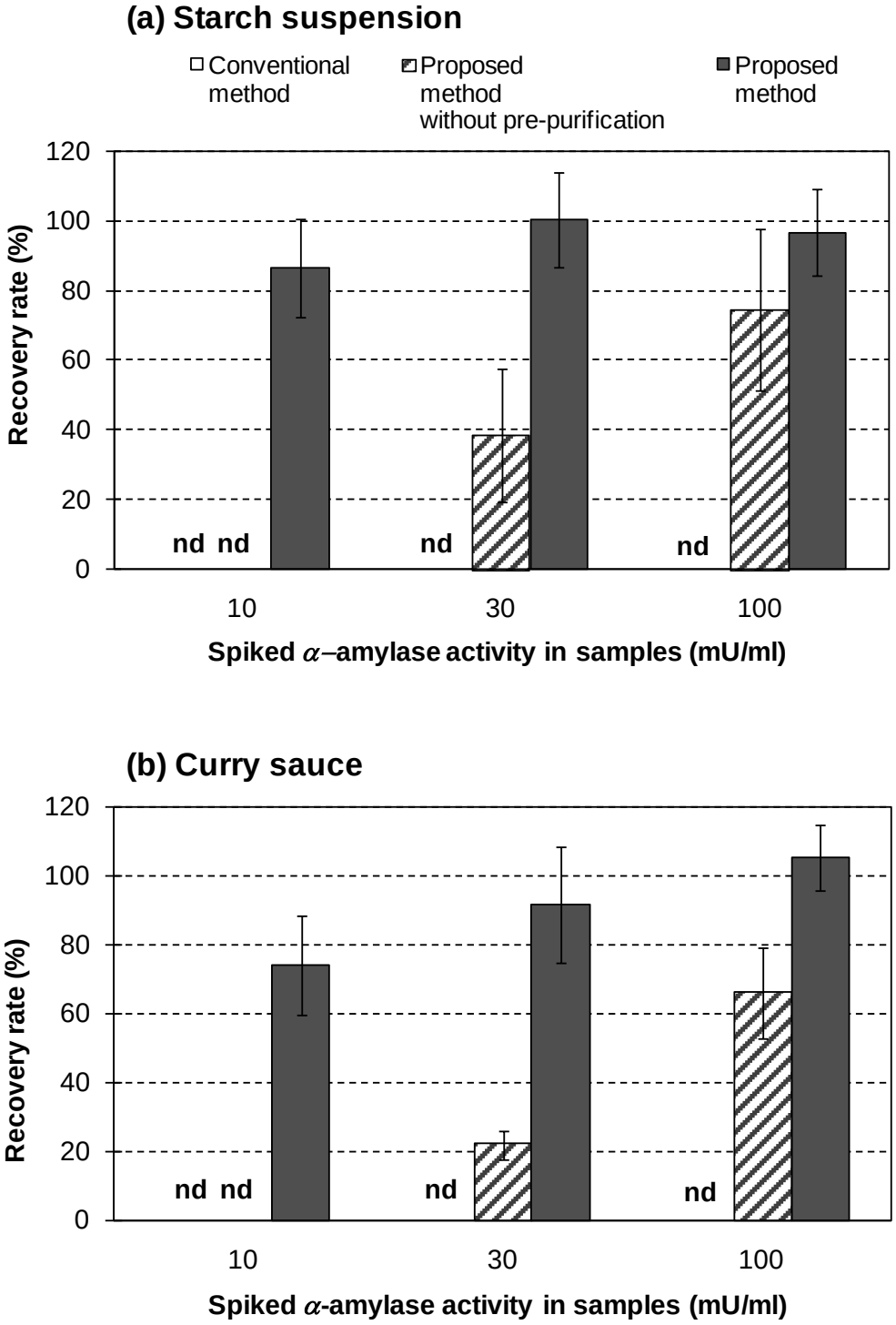

Figure 8. Recovery of α-amylase, expressed as the amount of activity relative to the activity of an α-amylase standard solution. Each method was performed in order to recover the α-amylase from (a) starch suspension and (b) curry sauce. Values shown are means ± SD obtained from five experiments.