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Microwave improvement of soy protein isolate–saccharide graft reactions

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Abstract

Soy protein isolate (SPI) was mixed with lactose, maltose, dextran or soluble starch (SS) and heated (temperature $\leqslant$90 °C) by microwave irradiation. Compared to the classical heating, the microwave heating (MH) speeded up the graft reactions of SPI with sugars. From the results calculated, the rate constants of free amino groups in the grafts of SPI with lactose, maltose, dextran and SS by MH were, respectively, 6, 7, 57 and 12.3 times of those by classical heating. After heating by microwave, the lysine and arginine decreased and from, the FTIR spectroscopy of SPI and its grafts by MH, the absorbance of –C–O stretching and –OH deformation vibrations (1050–1150 cm^{-1}) and free –OH form (3643–3630 cm^{-1}) in SPI grafts increased compared to SPI. The temporal development of the graft reactions of SPI with sugars by MH was also shown by SDS–PAGE.

Keywords: Microwave; Soy protein isolate; Saccharide; Graft reactions

1. Introduction

Many researchers have attempted to convert food proteins into useful proteins with better functional properties through physical, chemical and/or enzymatic treatments. However, most of these methods utilize toxic chemical products and are not permitted for potential industrial applications. Recently, some attempts were made to improve the functional properties of proteins through protein–saccharide graft reactions without any chemicals, which are based on Maillard-type reactions between the amino groups of protein and the reducing-end carbonyl groups of saccharide (Handa & Kuroda, 1999; Kato et al., 1988; Kato et al., 1990; Kato et al., 1991; Nakamura, Kato, & Kobayashi, 1991, 1992a, 1992b; Nakamura, Kobayashi, & Kato, 1994). It has been reported that protein–saccharide grafts are useful as a new functional biopolymer having excellent emulsifying, antioxidant, and antimicrobial effects for food applications (Handa & Kuroda, 1999; Kato et al., 1988, 1990, 1991; Nakamura, Kato, & Kobayashi, 1991, 1992a, 1992b).

There are two main ways reported to cause the protein–saccharide graft reactions. One involves protein–saccharide mixture, following dry heating at 60 °C, for a time period of tens of hours up to 3 weeks. Kato et al., 1990 reported that the safe ovalbumin–dextran conjugates can be prepared by covalent binding between the ε-amino groups in the protein and the reducing-end carbonyl group in the polysaccharide through a controlled Maillard reaction in the dry state without using any chemical reagents, but the conjugates of proteins with small carbohydrate molecules, such as glucose or lactose, under controlled dry heating, resulted in insoluble aggregates having poor surface properties. Interestingly, the emulsifying properties of the grafts of

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ovalbumin–dextran were much better than those of commercial emulsifiers, even at acidic pH or in high-salt conditions. In addition, other protein grafts with polysaccharides also showed a significant improvement in functional properties, such as emulsifying properties, solubility, antioxidative effect, and antimicrobial activity. For instance, insoluble wheat gluten was solubilized and its functional properties were improved by Pronase treatment followed by dextran (Kato et al., 1991). In the case of casein, the graft reaction of protein–polysaccharide was quickly done within 24 h (Kato, Mifuru, Matsudomi, & Kobayashi, 1992). On the other hand, it takes a long time to form protein–polysaccharide conjugates in the case of folded or rigid proteins, and the emulsifying properties of this conjugate were compared to those of commercial emulsifiers and found to be much better in acidic and high-salt conditions (Kato et al., 1992). Morever, Nakamura et al. (1991) reported that the lysozyme–dextran conjugate had bifunctional properties and excellent emulsifying properties with antimicrobial effects against both Gram-positive and negative bacteria. The other way involves a protein–saccharide (mainly monosaccharide and disaccharide) mixture in a buffer solution, following heating at a certain temperature for tens of minutes to several hours, which we call wet heating. For instance, β-lactoglobulin (BLG) and sereval common sugars (arabinose, galaetose, glucose, lactose, rhamnose or ribose) in mixture dissolved in phosphate buffer (pH 6.5) were put in tightly capped flasks and heated in a water bath at 60 °C for 72 h; through the reaction, the functional properties (solubility, heat-stability, emulsifying and foaming properties) of BLG were improved (Chevalier, Hobert, Popineau, Nicolas, & Haertle, 2001).

Soy proteins serve as an ingredient in many food formulations. Although soy protein ingredients perform many functions in food systems in which they are incorporated, they are best known as emulsifying and gelling agents. In particular, they can also be grafted with polysaccharides such as cellulose, resulting in a hybrid which possesses improved emulsifying properties (Diftis & Kiosseoglou, 2003). The present study focusses on the graft reaction of soy protein isolate (SPI) with several sugars, which was carried out by a new procedure using microwave heating (MH), in order to improve the reaction rate. Previous researches on protein–saccharide graft reactions or Maillard reactions through dry heating or wet heating methods gave very good results, however, in general, it took a long time, such as tens of hours (wet heating) or several weeks (dry heating), to form the protein–saccharide grafts. Therefore, in our research, a new procedure, microwave heating, was applied for the reactions but the graft reactions or Maillard reactions of protein with sugars by MH have scarcely been reported until now.

2. Materials and methods

2.1. Materials

Soy protein isolate (SPI) was supplied by Fuji Oil (Japan) and analyzed for protein (91%, $N \times 6.25$) by the Kjedhal method. Lactose, maltose, dextran and soluble starch (SS) were obtained from Shanghai Chemical Co. (Shanghai, China). All other reagents were of analytical grade. The microwave oven used for heating was a Panasonic (NN-S563JF Model, 1000 W full power). The centrifuges ware (Cat. No. 3119-0050, NALGENE, USA), used for graft reactions, height 106.7 mm, outer diameter 28.8 mm, volume 50 ml, were made of PPCO (Polypropylene Copolymer).

2.2. Graft reaction by classical heating

SPI and sugar were dissolved in phosphate buffer (1/15 M, pH 7.8) to give a weight ratio of sugar to SPI of about 2:1 (for lactose and maltose) or 5:1 (for dextran and SS). The protein concentrations were estimated using the Bradford method (Bradford, 1976). And then the mixtures of protein and sugar were put in a tightly capped centrifuge ware and heated in a water bath at 90 °C. The reported heating time included the heating up period of about 2–3 min. After a given heating time, samples were cooled in ice water, prior to analysis. The reaction mixtures were heat-treated at least in duplicate.

2.3. Graft reaction by microwave heating

The graft reaction by microwave heating (MH) was carried out in a microwave oven (Panasonic), and power level used was 1000 W. SPI and sugar were dissolved in phosphate buffer (1/15 M, pH 7.8) to give a weight ratio of sugar to SPI of about 2:1 (for lactose and maltose) or 5:1 (for dextran and SS). The protein concentrations were also estimated using the Bradford method (Bradford, 1976). And then the mixtures of protein and sugars were put in a tightly capped centrifuge ware and heated by the microwave irradiation for 1.5 min to 90 °C and then cooled in an ice bath to stop the reaction for 3 min (the temperature of mixture was about 21 °C); after that, the above was repeated for several times. The total heating time was calculated as $1.5 \times N$ min, N, being the number of repititions. After the reaction, samples were cooled in ice water, prior to analysis. The reaction mixtures were heat-treated at least in duplicate.

2.4. Determination of free amino groups

The quantity of available amino groups was determined by the modified *ortho*-phthaldialdehyde (OPA)

method (Church, Swaisgood, Porter, & Catignani, 1983; Nielsen, Petersen, & Dambmann, 2001). The OPA reagent was prepared daily by mixing 40 mg of OPA (dissolved in 1 ml of methanol), 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% (w/w) sodium dodecyl sulfate (SDS) and 100 μl of β-mercaptoethanol, and then diluting to a final volume of 50 ml with water. 100 μl of sample solution were added to 2 ml of OPA reagent. The solution was mixed briefly by inversion and incubated for 2 min at ambient temperature, and the absorbance was read at 340 nm in a UNICO UV-2100 spectrophotometer. A calibration curve was obtained by using 0.25–2 mM L-leucine as a standard.

2.5. Analyses of browning

Browning of SPI–saccharide mixture by the graft reaction was measured spectrophotometrically as absorbance at 420 nm (Brands, Wedzicha, & van Boekel, 2002). The samples were also diluted twice in 20% SDS (W/W) to reduce light scattering. The blank value obtained from an unheated sample was subtracted from the sample reading.

2.6. SDS–PAGE

The reaction mixtures were analysed by SDS–PAGE (Laemmli, 1970) on 10% (w/v) acrylamide separating gel and 4% (w/v) acrylamide stacking gel. Samples were prepared in Tris–HCl of pH 8.0, containing 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol. In order to enhance detection sensitivities of proteins, a combined Coomassie blue-silver stain method (De Moreno, Smith, & Smith, 1985; Zhu, Wang, & Yu, 1999) was applied: the gel sheets were fixed in 50:10:40 methanol:acetic acid:-H_2O stained for protein with Coomassie brilliant blue G-250 and then stained with silver.

2.7. Gel filtration of grafts by MH

SPI–saccharide grafts were separated by gel filtration on a SepharoseCL6B column (65 × 2 cm) equilibrated and eluted with 50 mM HCl–Tris buffer (pH 8.0) at a flow rate of 20 ml h^{-1}. The protein content in each fraction was detected by measuring the UV absorbance at 280 nm. The void volume fraction cotaining the SPI–saccharide grafts was collected, dialyzed (with a cut-off value of 12,000 Da) against distilled water at 4 °C for 48 h, and lyophilized. The resulting grafts were stored at 4 °C.

2.8. Analysis of amino acids

The lysine and arginine of SPI and grafts separated by gel filtration were determined by an HP1100 system (Agilent Co., USA) for amino acid analysis after acid hydrolysis at 110 °C for 24 h in 6 N HCl in evacuated sealed tubes (Matsuda, Kato, & Nakamura, 1991).

2.9. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra of SPI and grafts separated by gel filtration were recorded using a Ntcolet 5DXC spectrometer (Ntcolet Co., USA) from 4000 to 400 cm^{-1} using 4 cm^{-1} resolution and an accumulation of 32 scans.

2.10. Calculation of reaction rates

Based on the general rate law (Martins, Jongen, & van Boekel, 2001), the disappearance of a compound is

$$\frac{dA}{dt} = kA^n, \tag{1}$$

in which the decrease in concentration of component A over time t is related to the concentration of that component, where k is the reaction rate constant and n (usually $0 \leqslant n \leqslant 2$) the reaction order. By integration of the differential equation to a chosen order, with respect to time, a zero-order reaction would be

$$A = A_0 - kt. \tag{2}$$

A first-order reaction would be

$$A^{-1} = A_0 e^{-kt}. \tag{3}$$

And a second-order reaction would be

$$A^{-1} = A_0^{-1} + kt, \tag{4}$$

in which A_0 is the concentration of component A at time 0. In this study, A is the concentration of free amino groups.

According to the equations above, the reaction order could be decided from the data obtained, and therefore the reaction rate constant k of free amino groups in protein could be calculated.

3. Results and discussion

3.1. Preliminary study

Protein–saccharide graft reactions are based on the Maillard-type reaction, but need much more time to take place using classical heating, such as water baths, oil baths, sand baths and heating jackets, which might be difficult in application. Since the mid-1990s, microwave heating (MH) for organic chemistry has increased significantly, the main reason for this is mostly due to an increased interest in shorter reaction times (Lidstrom, Tierney, Wathey, & Westman, 2000). The graft reactions of proteins with sugar belong to organic synthesis,

and the MH might shorten the reaction times. Therefore, in this study, we introduce the MH technique to assist the graft reactions of SPI with sugars. In general, polar molecules, heated under microwave irradiation and water can, thus, behave as a pseudo-organic solvent at elevated temperatures (Lidstrom et al., 2000). So wet heating was chosen, and from the reports on the protein–saccharide reactions using wet heating (Brands et al., 2002; Chevalier et al., 2001), the reaction mixtures are often in a closed system to avoid possible oxidation of samples and variation. Accordingly, a tightly capped centrifuge ware was employed, filled with reaction mixture and placed in the centre of the microwave oven at a marked place, and the procedure of MH at intervals was used to control the reaction temperature, which is, by controlling the time of MH and the power of microwave, to keep the maximal temperature of reaction at a certain level, and then cooling the reaction mixtures in the ice bath to stop the reaction, and whereafter repeat it. Consequently, not a microwave reactor, but a Panasonic domestic household oven, was employed in this work, only to heat the reaction mixtures.

The reaction temperatures by MH were immediately determined by a mercury thermometer when the heating was for a certain time in the preliminary tests, in order to decide the time of MH. At the 1000 W level, the temperatures of the mixtures of SPI with all studied sugars rose rapidly to about 90 °C (Fig. 1), which implied that the different sugars had no different effects on heating up in the microwave oven. So the heating time from 21 to 90 °C by MH was 1.5 min at a time, and then the samples could be cooled to 21 °C in 3 min through the ice bath, which was a reaction cycle, and repeated several times. Since the system could allow place thermal transfer to air during measuring the temperature, there may be a slight deviation from the real temperature values,

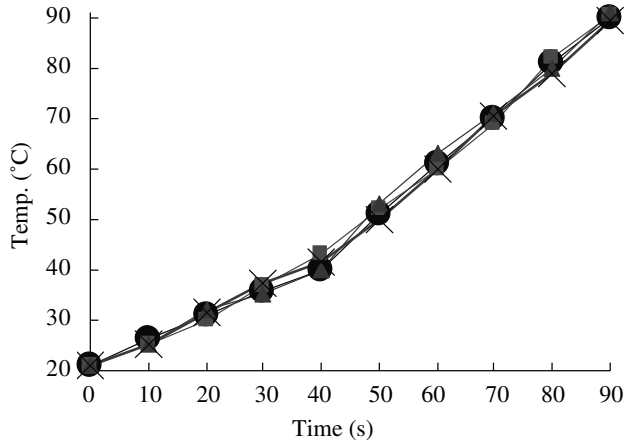

Fig. 1. Temperature rise in a centrifuge ware, containing the mixtures of SPI with lactose (●), maltose (■), dextran (×) or SS (▲) heated in a microwave oven at an effect of 1000 W.

3.2. Free amino acids and browning

When SPI–lactose, maltose, dextran or SS mixtures in phosphate buffer were heated at 90 °C, a brown colour development took place after a certain time of heating which was based on the Maillard-type reaction, possibly between the free amino groups of protein and the reducing end group of sugars, and the colour intensity increased thereafter, but the concentrations of free amino groups of soy protein decreased (Figs. 2 and3). When the concentrations of free amino groups in the protein grafted with the same sugar such as lactose (Figs. 2(A) and 3(A)), reached the same level, the reaction time needed by the classical heating or the water bath was much greater than that by MH, and on the degrees of browning of the mixtures, the same results were also obtained. It was very interesting, however, that the degrees of browning of SPI–lactose or maltose using classical heating were much more than those using MH as the concentrations of free amino groups fell to the same level, but the same phenomenon did not appear in the graft of SPI–dextran or SS, which perhaps indicated that there could be a side reaction, caramelisation (Homoki-Farkas, Orsi, & Kroh, 1997). This could produce brown colours during the reaction; therefore, the browning determined might arise from graft and/or caramelisation. In the polysaccharide solutions, the number of reducing end groups was less than that in the disaccharide solutions, so the disaccharides underwent caramelisation more easily, especially when heating for a long time such as when using classical heating, microwave heating could reduce the side reactions occurring during the graft reaction of SPI–saccharide. Moreover, by the same heating method, the graft reactions of SPI with lactose (Fig. 2(A) and 3(A)) or maltose (Figs. 2(B) and 3(B)) occurred more easily than that with dextran (Fig. 2(C) and 3(C)) or SS (Fig. 2(D) and 3(D)) in that the latter took longer to reach the same level. In addition, in both heating methods, there were also different results obtained for SPI with lactose and maltose, possibly due to different conformations in the molecular structure. This is maybe due to the molecular structures affecting the reaction processing. But, when SPI was heated without sugars by MH and classical heating, the concentrations of the free amino groups in the protein were not changed (Table 1), which implied that the microwave heating, to the same temperature as classical heating, could not destroy the protein by hydrolysis.

3.3. Electrophoresis

Coomassie Brilliant Blue staining is widely employed in electrophoresis, and requires an acid environment to enhance ionic interactions between the dye and the basic amino acid moieties of the protein, as well as to augment

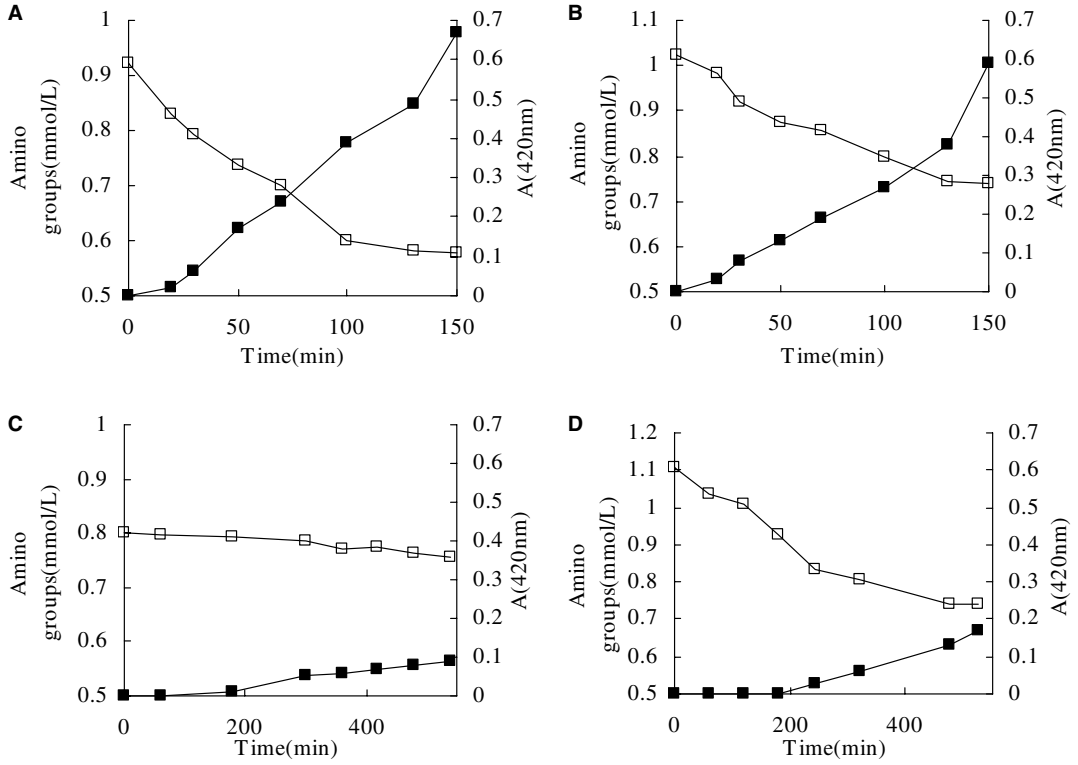

Fig. 2. Temporal development of free amino groups (□) and browning (■) of SPI with: lactose (A); maltose (B); dextran (C); SS (D) heated in a water bath at 90 °C.

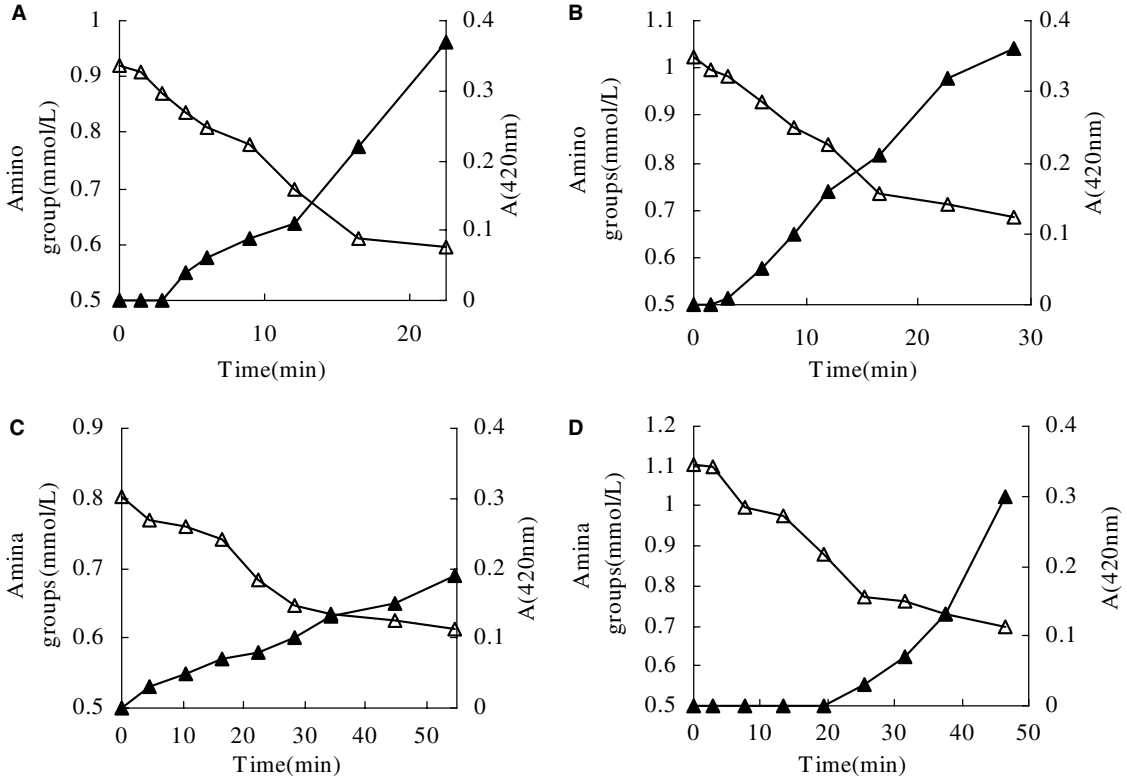

Fig. 3. Temporal development of free amino groups (△) and browning (▲) of SPI with: lactose (A); maltose (B); dextran (C); SS (D) heated at interval in a microwave oven at an effect of 1000 W. The temperature of the reaction system was not more than 90 °C.

Table 1
Free amino groups of SPI without sugars by MH and classical heating at 90 °C

Time (min)	0	15	30	60
MH (mM)	1.02 ± 0.02	1.01 ± 0.02	1.02 ± 0.01	ND
Classical heating (mM)	1.02 ± 0.02	ND	1.01 ± 0.01	1.02 ± 0.02

ND, not detected.

secondary dye–protein interactions due to hydrogen bonding, Van der Waals attraction and hydrophobic bonding (Wirth & Romano, 1995). However, the free amino groups of protein were reduced in protein–saccharide graft reactions and saccharide molecules were linked to the protein in the grafts, so the interactions of the dye and the protein could be affected and thus decrease the detection sensitivities. According to De Moreno et al. (1985) and Zhu et al. (1999), a combined Coomassie blue-silver stain method was choosen in this research to enhance the detection sensitivities of proteins.

In Fig. 4 the SDS–PAGE patterns of soy protein isolate–saccharide mixtures are presented for various heating times of microwaves. The mixtures by MH resulted in the disappearance of bands of subunits and, after a certain time (for each mixture) of the reaction, the electrophoretic bands could not been seen in Fig. 4, such as about 12 min for the SPI–lactose reaction (Fig. 4(A)). Similar electrophoretic patterns were observed by Diftis and Kiosseoglou (2003) for SPI–NaCMC mixtures, but in the researches of Diftis and Kiosseoglou (2003) and other workers on protein–polysaccharide mixtures (Kato et al., 1992; Kato, Minaki, & Kobayashi, 1993;

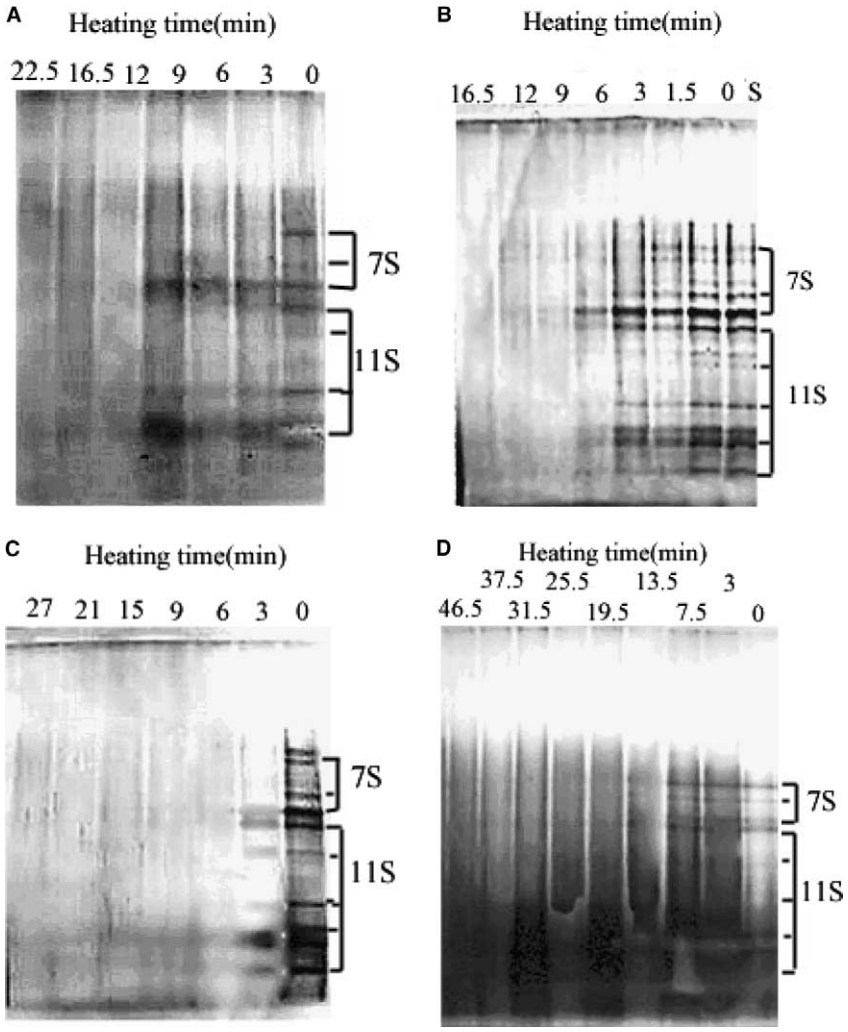

Fig. 4. SDS–PAGE patterns for the temporal development of the graft reactions of SPI with: lactose (A); maltose (B); dextran (C); SS (D) by MH. S stands for the SPI without sugars by MH for 30 min.

Matsudomi, Tsujimoto, Kato, & Kobayashi, 1994), there were broad bands near the tops of the stacking and the separation gels, indicating a wide distribution of molecular weights of the products of the reaction which might consist of polymerized protein molecules due to isopeptide (Diftis & Kiosseoglou, 2003), while similar bands were not found in this research, even for SPI–dextran or SS mixtures, which might account for no covalent polymerization produced between protein molecules but a covalent complex of protein with sugars in the reaction by MH. It was reported that the interactions of SPI–polysaccharide were mainly confined to subunits of 7S and the acidic subunits of the 11S fraction of the protein, and the acidic subunits of the 11S fraction were less reactive, possibly due to their much lower lysine content (Difits et al., 2003). Similar results are also shown in Fig. 4. By microwave heating, the acidic subunits of the 11S fraction also reacted with sugars, and even polysaccharides such as dextran and SS. MH might not only speed the rates of SPI–saccharide graft reactions, but also elevate the degree of the reactions. However, the electrophoretic bands became blue red or dark with increase of molecular weight of sugars in the graft by MH (Fig. 4), which could indicate that the distribution of molecular weights of the products of SPI–polysaccharide graft had been changed because polysaccharides were linked to the protein molecules by covalent bond, e.g., SPI–SS (Fig. 4(D)). In addition, the electrophoretic pattern of SPI without sugars by MH is also shown in Fig. 4(B), and there is no change compared to the SPI without heating, which also implies that the microwaves could not destroy protein by hydrolysis and might indicate that microwave irradiation could alter protein conformation (de Pomerai et al., 2003) to improve the SPI–saccharide graft reaction.

3.4. Reaction rates of free amino acids

In order to understand the graft reaction of SPI with the sugar, we carried out the fit of the kinetic model of Eq. (1) to the experimental data, and found that the change of the free amino groups of SPI–saccharide with heating time accorded with the first order model of Eq. (3) very well. The results are shown in Table 2. From the results calculated, the rate constants of free amino groups in the grafts of SPI with lactose, maltose, dextran or SS by MH were, respectively, 6, 7, 57 and 12.3 times those by classical heating. So, microwave irradiation is able to speed up the graft reactions of SPI–saccharides, but the the reaction rate increase depended on the varieties of sugars, which took part in the reaction.

Since the introduction of microwave-assisted organic synthesis in 1986, the main advantage is the shorter reaction times (Lidstrom et al., 2000). The ratio of the reaction can be described by an Arrhenius relationship as follows (Lidstrom et al., 2000):

$$K = A\mathrm{e}^{\Delta GIRT}. \quad (5)$$

Considering the above equation, Lidstrom et al. (2000) reported that there were basically two ways to increase the rate of a chemical reaction. First is the pre-exponential factor A, which describes the molecular mobility and depends on the frequency of vibrations of the molecules at the reaction interface. It is interesting that microwave can induce an increase in molecular vibrations and it has been proposed that this factor, A, can be affected. Another possible reason is, that microwave irradiation produces an alteration in the exponential factor by affecting the free energy of activation, ΔG. In this study, microwaves possibly determined the molecular vibrations of protein and sugars, so the reaction rates were improved by using MH. Because the protein in the reaction was the only SPI, the molecular mobilities of sugars might be the reason that the graft reactions of SPI with different sugars had the different reaction rates. Thus, the reaction rates of SPI–saccharide were different, and the reactions of SPI with SS and dextran, which were inhibited when reacting with SPI using classical heating due to fewer reducing end groups than the lactose and maltose, were considerably speeded up by MH, especially for SPI–dextran. This suggests that microwaves markedly altered the molecular mobility of dextran.

3.5. Amino acids and FTIR spectroscopy

Table 3 shows the contents of lysine and arginine of the grafts of SPI–lactose, maltose, dextran and SS using MH, as measured by amino acid analysis after the acid hydrolysis. The lysine and arginine were both decreased after heating SPI with sugars for a given time, which

Table 2
Rate constants of the free amino groups disappearance in the grafts as found by applying the first kinetic model of Eq. (3) to the data of SPI–saccharide systems (90 °C, pH 7.8) by classical heating and microwave heating, using least-squares criterion ($p < 0.05$)

Reaction systems	Classical heating		Microwave heating	
	Rate constant	R square	Rate constant	R square
SPI–lactose	0.0036	0.9291	0.0212	0.9693
SPI-maltose	0.0024	0.9598	0.0160	0.9574
SPI–dextran	0.0001	0.9097	0.0057	0.9117
SPI–SS	0.0009	0.9396	0.0111	0.9553

Table 3
Lysine and arginine contents of the grafts of SPI with lactose, maltose, dextran and SS by MH for a given time

Amino acid	SPI (0 min)	SPI–lactose (22.5 min)	SPI–maltose (12 min)	SPI–dextran (45 min)	SPI–SS (37.5 min)
Lysine (%)	6.25	3.45	5.89	5.75	4.19
Arginine (%)	7.40	5.80	6.90	6.10	5.82

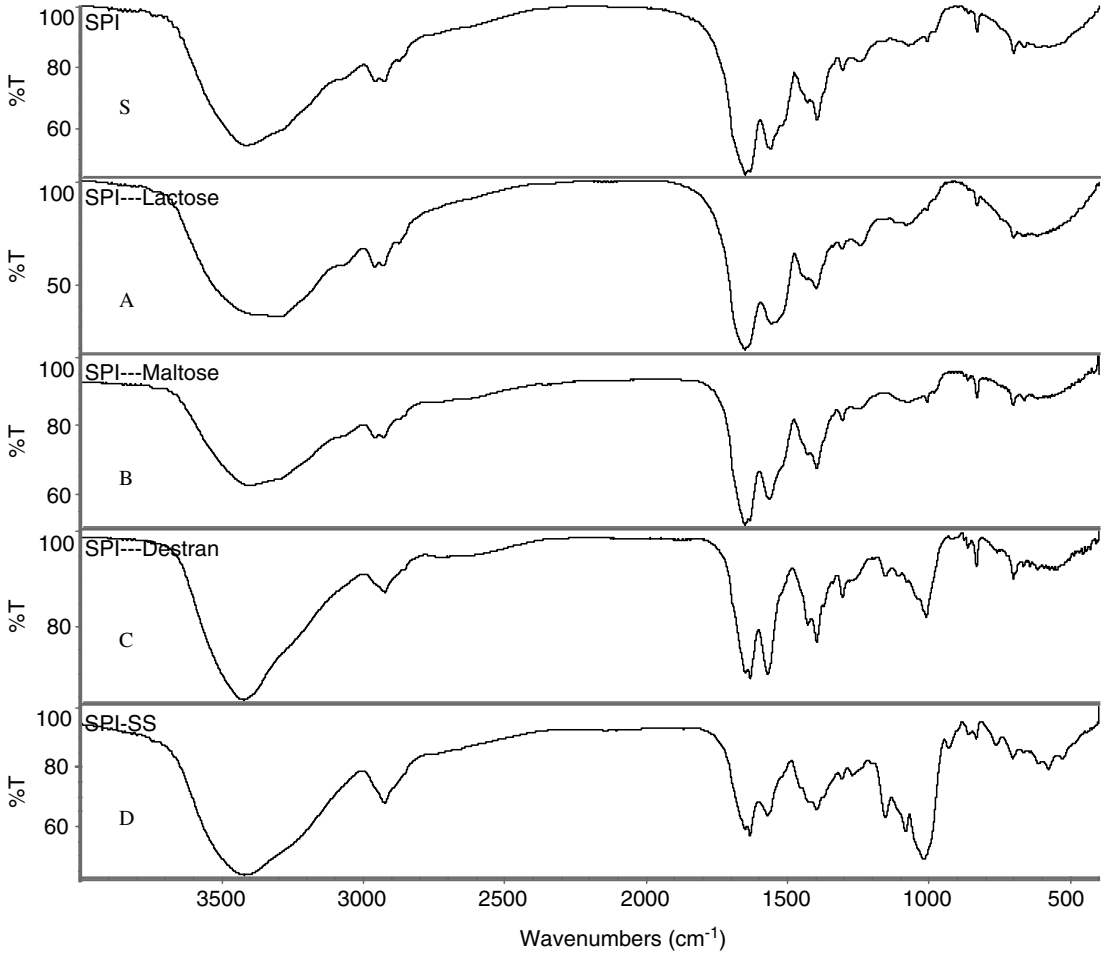

Fig. 5. FTIR spectroscopy of SPI (S) and its grafts with lactose (A), maltose (B), dextran (C) and SS (D) by MH.

suggested that the free amino groups taking part in the reaction mainly came from lysine and arginine of the protein. In addition, from the FTIR spectroscopy of SPI and its grafts by MH (Fig. 5), the absorbance of –C–O stretching and –OH deformation vibrations (1050–1150 cm^{-1}) and free –OH form (3643–3630 cm^{-1}) in SPI grafts by MH increased compared to SPI, especially for polysaccharides, dextran and SS, which also indicated that saccharide molecules were linked to the protein by the covalent bonds.

From the results above, the grafts of SPI with sugars using MH were formed by covalent binding between the ε-amino groups in the protein and the reducing-end carbonyl groups in the saccharide, which is similar to protein–saccharide graft reactions through other heating methods (Kato et al., 1990, 1992).

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