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ENZYMATICALLY MODIFIED SOY PROTEIN
Part I. Thermal behaviour

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Abstract

Optimum temperature and pH for the isolation of soy protein isolate (SPI) from soy protein concentrate (SPC) were established. Enzymatic hydrolysis of SPI with enzymes of different specificities such as trypsin, chymotrypsin, papain and urease was carried out and the products of hydrolysis were characterized by molecular mass determination [sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)] and thermal techniques [differential scanning calorimetry (DSC) and thermogravimetric analysis (TG)]. Enzymatic hydrolysis resulted in a significant reduction in molecular masses. However the thermal stability of hydrolysed SPI was similar to native SPI indicating that it is independent of molecular mass. DSC studies indicated an increase in temperatures of endothermic transition associated with SPI denaturation and loss of absorbed moisture in samples of lower molecular masses.

Keywords: chymotrypsin, DSC, papain, SPC, SPI, TG, trypsin

Introduction

The studies on soy proteins and their applications in industrial products such as adhesives for wood and paper, binders in coatings and paints and as emulsifiers in colloidal rubber products were carried out extensively during early part of the twentieth century and are well documented in [1–3]. However, the availability of petroleum at a lower cost and biochemical inertness of petroleum-based products proved disastrous for the industrial use of soy protein. It is only after a lapse of almost 50 years that the significance of eco-friendly materials has been realized and once again, the polymer scientists are looking at biopolymers. There is a need to develop eco-friendly polymeric materials using renewable resources. Plywood industry needs environment-friendly adhesives from renewable resources because petroleum resources are finite and are becoming limited, whereas the demand for adhesives is increasing [4].

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Abundant proteins are available from renewable resources and agricultural processing by-products such as soybean proteins from oil processing. Utilization of these protein by-products as biodegradable adhesives and resins will help to overcome environmental problems and add value to agricultural by-products. Soy-based products are now being investigated as plastics, adhesives, films and coatings [1, 4–6].

Enzymatic modifications, which involve minimum side reactions and lead to limited hydrolysis of selected peptide bonds, have been used to improve functionalities of protein [7, 8]. Partial enzymatic hydrolysis increases solubility, foaming and emulsifying property [9]. Proteolytic enzymes are generally used for such modification as they hydrolyse specific peptide bonds.

Very few studies on enzymatic hydrolysis of soy protein isolate and its effect on functional properties have been reported [10, 11]. It would be of interest to investigate the modification of soy protein isolate using enzymes with different specificity. We now report the modification of soy protein isolate using trypsin, chymotrypsin, papain and urease. Trypsin (pH 8) hydrolyses peptide bonds whose carbonyl function is donated by basic amino acids such as lysine or arginine. Chymotrypsin (pH 8) catalyses most effectively the hydrolysis of the peptide bonds on carboxyl side of amino acid residues with large hydrophobic side chains (e.g. phenylalanine, tryptophan or tyrosine) [12]. Papain is noted for its wide specificity. It preferentially cleaves peptide bonds involving basic amino acids, particularly arginine, lysine and residues following phenylalanine [13].

The products of enzymatic hydrolysis are generally characterized by chromatography or electrophoresis. Thermal characterization techniques, such as differential scanning calorimetry (DSC) or thermogravimetry (TG), which are extensively used for determination of phase transitions, thermal stability and curing behavior of synthetic polymers, are rarely used in proteins or biopolymers [14]. The possibility of using DSC for studying the protein modification during enzymatic hydrolysis (using *cucurbita* and *pomiferin*) of soy protein isolate has been reported only recently [15]. Thermal behaviour (T_g and crystallization) of seed prolamine films from rice, wheat and soybean has been reported recently [16]. Studies on effect of water on T_g of Nile Tilapia myofibrillar proteins (100 g) and glycerol (70 or 30 g) have also been carried out [17]. However, so far no systematic studies using DSC and TG techniques for the characterization of products of enzymatic hydrolysis have been reported.

In the present work, the characterization of the products of the hydrolysis of soy protein was done by molecular mass determination and thermal techniques (DSC and TG). The objective of the work was to establish a correlation between molecular mass changes with thermal transitions and stability of soy protein after enzymatic hydrolysis.

Materials and methods

Materials

Soy protein concentrate (SPC) was procured from general health store containing 73% protein on dry basis. Defatted soy meal was purchased from Hi media laboratories,

Mumbai. Chemicals used were acrylamide, glycerol, 2-mercaptoethanol (Spectrochem), N,N^1-methylene-bis-acrylamide (BIO-RAD), sodium dodecyl sulfate (SDS), glycine, bromophenol blue (Qualigens), tris (hydroxy-methyl)aminomethane (Tris) (SRL), coomassie brilliant blue R 250 (Merck, electrophoresis grade), N,N,N^1,N^1-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), (both ACS grade). Markers (Banglore GENEI), bovine serum albumin (BSA, Merck), Folin's Lowry reagent (Spectrochem), enzymes – trypsin (7500 BAEE U mg^{-1}), papain (10–20 U mg^{-1}), urease (from Jackbean) (all CDH), chymotrypsin (40–60 U mg^{-1}), (Sigma chemicals), and biocide (benzisothiazole and chloromethylisothiazoline) supplied by ICI paints were used as received.

Preparation of soy protein isolate (SPI) from soy protein concentrate (SPC)

In order to optimize the conditions for the preparation of SPI from SPC, the effect of temperature and pH was investigated. SPC was solubilised at 22°C at different pH. In a typical process 2 g of SPC was dispersed in 25 mL of distilled water in a conical flask. Two percent of biocide was added and pH was adjusted to 7–11 by using 1.0 M NaOH. The dispersions were then stirred for 1 h using a magnetic stirrer at 22°C for thorough mixing. The suspension was then centrifuged on Rota-4R Centrifuge (Plastocraft Industries, Mumbai) at 4000×g for 10 min to remove the carbohydrates and insoluble proteins. The soluble protein was decanted and used for characterization i.e to determine the protein solubility. The effect of temperature was investigated at 22, 30, 37, 45 and 55°C at pH 11 using similar procedure mentioned above. Protein solution thus obtained was then solidified at –72°C and lyophilized in Eyella Tokyo freeze dryer FD-1.

Preparation of 7S-RG and 11S-RG fractions

7S and 11S-RG (Rich Globulin) was isolated from defatted soy meal according to the method described by Nagano *et al.* [18]. The 11S-RG was the protein precipitated by adjusting the pH of defatted soy meal to 6.4 and leaving the dispersion for 12 h at 0–4°C. After the isolation of 11S-RG fraction of protein, 7S-RG fraction was precipitated at pH 4.8. Both the protein fractions were solidified at –72°C and lyophilized as described earlier.

Enzymatic modification of soy protein isolate

Four samples of SPI (10 mL each) having 7% (mass/vol) solid content and the enzymes i.e. papain, trypsin, urease, chymotrypsin in the ratio of 50:1 were mixed at pH 8. The enzyme-catalyzed hydrolysis of the substrate was carried out at 37°C for 2 h under shaking (200 rpm) conditions. The hydrolysates were taken out after an interval of 1 and 2 h and inactivated by keeping in boiling water bath for 5 min. All enzyme-modified SPI were lyophilized before further studies. These hydrolysates have been designated by writing a prefix (indicating the enzyme) to SPI. For example the product of hydrolysis of SPI using urease or papain has been designated as USPI and PSPI, respectively.

Characterization techniques

Protein solubility

A Uvikon spectrophotometer 930 (Kontron) was used to determine the absorbance at 750 nm. The protein content of SPI was determined according to Lowry's method. A calibration curve was obtained by plotting absorbance (750 nm) *vs*. concentration of pure protein (bovine serum albumin). The absorbance of soy protein isolate obtained at different pH and temperature was recorded. The protein content was obtained from the calibration curve.

Solid content

Solid content of the SPI isolate was found by heating the solution in an air oven at 80°C for about 12 h or till a constant mass was obtained.

Clarity

The clarity of SPI solution obtained from SPC dispersion at different pH was determined by taking absorbance of SPI solution at 660 nm using double distilled water as blank [19].

Molecular mass determination

Molecular mass of SPI, 7S-RG, 11S-RG and all enzyme modified SPI was determined by using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Laemmli [20]. Protein samples (10 μg μL^{-1}) were prepared in a buffer containing 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 15% glycerol, 5% 2-mercaptoethanol and 0.25% bromophenol blue. Standard markers having molecular mass of 29 kDa (carbonic anhydrase), 43 kDa (ovalbumin), 68 kDa (bovine serum albumin, BSA), and 97.4 kDa (phosphorylase) were used for comparing the molecular mass of protein samples. 10 μL of each protein samples was applied in the wells of the stacking gel. Stacking gel (pH 6.8) and separating gel (pH 8.8) were 5 and 8%, respectively. After electrophoresis, the gels were stained with 0.25% coomassie blue, R-250 and destained until the background stain was removed.

Thermal study

TA 2100 thermal analyser having a 910 DSC module and 951 TG module was used for the thermal characterization of SPI, SPC, 7S-RG, 11S-RG fractions and all enzyme modified SPI. DSC scans were recorded in static air atmosphere at a heating rate of 10°C min^{-1} by using 5±1 mg of powdered samples. Thermal stability was determined by recording TG/DTG traces in nitrogen atmosphere (flow rate=60 cm^3 min^{-1}) using powdered samples. A heating rate of 10°C min^{-1} and the sample mass of 10±1 mg was used in each experiment.

Results and discussion

Protein solubility

The unfolding of proteins, which affect their solubility, is dependent on pH and temperature. As the pH of the SPC dispersion was increased from 7 to 11 the yield of SPI increased from 20±2 to 35±2% (Fig. 1). Increase in temperature also enhanced the isolation of SPI from SPC. An increase in protein content from 35±2 to 57±2% was observed on increasing the temperature from 22 to 55°C (Fig. 2) at optimum pH i.e. 11. On the basis of these studies it was concluded that optimum pH and temperature for isolation of SPI are 11 and 55°C, respectively.

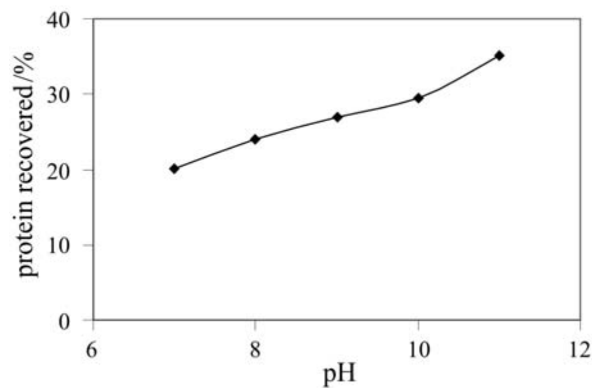

Fig. 1 Effect of pH on recovery of SPI from SPC

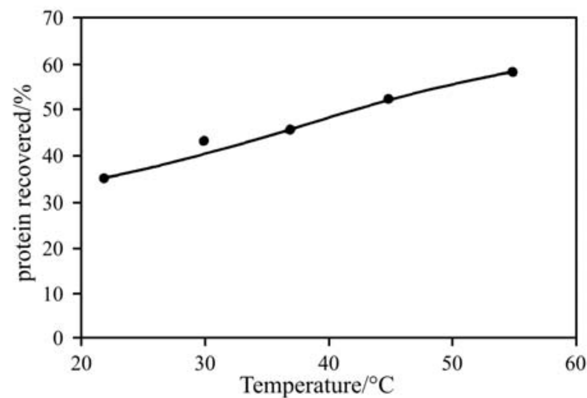

Fig. 2 Effect of temperature on recovery of SPI from SPC

Clarity

62% increase in clarity of the SPI solution was observed as the pH increased from 7 to 9. At pH 10 and 11, the clarity of SPI solution remained almost constant (Fig. 3).

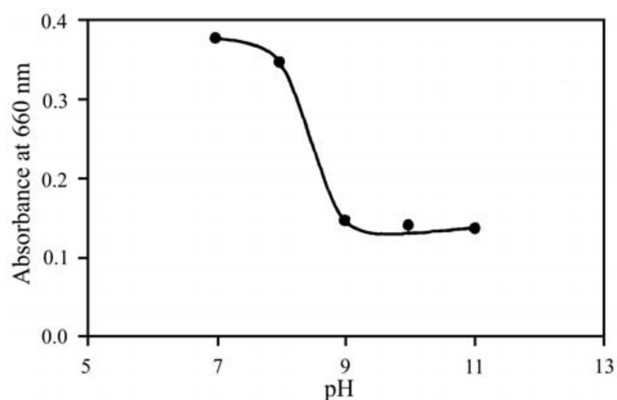

Fig. 3 Effect of pH on clarity of SPI

Molecular masses

An analysis of total protein from SPI showed mixture of high and low molecular mass proteins shown in the form of a continuous band in the gel. However, two distinct molecular mass fractions of 35–39 and 125–141 kDa were resolved, which signified that the protein fraction of these molecular masses were predominant in soy protein (Fig. 4, lane 2). 7S-RG fractions had four different molecular mass fractions 79–83, 63–70, 50–52, 35–39 kDa (Fig. 4, lane 3). 11S-RG fraction of SPI showed 5 fractions of different molecular masses i.e. 79–83, 63–70, 50–52, 44–46, 35–39 kDa (Fig. 4, lane 4). The band of very high intensity at 35–39 kDa represented the acidic subunit of 11S-RG protein. α, α^1 and β subunit of 7S-RG are denoted by 79–83, 63–70 and 50–52 kDa molecular mass bands respectively. SPI obtained from SPC contained the acidic subunit of 11S-RG fractions. Comparing the molecular mass bands of 7S-RG and 11S-RG

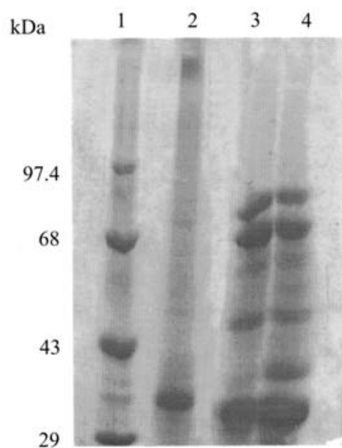

Fig. 4 Electrophoretic profiles of SPI, 7S-RG, 11S-RG. Lane 1 – Molecular mass standard; Lane 2 – SPI; Lane 3 – 7S-RG; Lane 4 – 11S-RG

fractions with the SPI, it was observed that in 7S-RG and 11S-RG fraction high molecular mass proteins were not present but there was an overlapping of bands in 7S-RG and 11S-RG, which, may be due to contamination [21, 22]. Moreover, the intensity of continuous molecular mass bands higher than 97.4 kDa was missing in the 7S-RG and 11S-RG fractions. Sun *et al.* [23] have earlier reported the SDS-PAGE pattern of 7S-RG and 11S-RG fractions indicating that 7S-RG and 11S-RG had highly stained bands for their corresponding subunits plus some minor bands due to contamination.

Chymotrypsin modified soy protein isolate (CSPI) showed only one molecular mass band which was of 23–25 kDa size and of low intensity, which is the basic subunit of 11S-RG fraction (Fig. 5, lane 3). All other molecular mass bands present in SPI were absent. This suggested extensive hydrolysis of SPI by chymotrypsin in 1 h. In urease modified SPI (USPI), α, α^1 and β subunit of 7S-RG molecular mass bands were absent (Fig. 5, lane 6).

High molecular mass bands of 63.4–79.4 and 85–89 kDa were absent in trypsin modified SPI (TSPI) (Fig. 6, lane 3). Increase in the duration of hydrolysis to 2 h, resulted in a decrease in intensity of the bands (Fig. 6, lane 4). Papain modified SPI (PSPI) showed extensive hydrolysis after 1 h duration and all the significant molecular mass bands were absent with the exception of 23–25 kDa band (Fig. 7, lane 5). But after 2 h of hydrolysis, this basic subunit band of 11S-RG also disappeared (Fig. 6, lane 6). Unlike

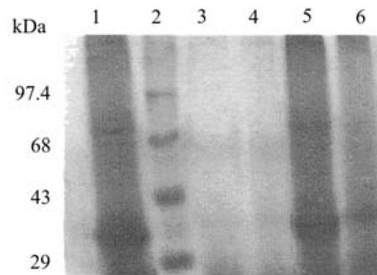

Fig. 5 Electrophoretic profiles of CSPI, USPI. Lane 1 – SPI; Lane 2 – Molecular mass standards; Lane 3 – CSPI after 1 h; Lane 4 – CSPI after 2 h; Lane 5 – SPI; Lane 6 – USPI after 2 h

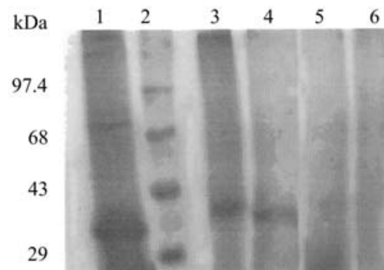

Fig. 6 Electrophoretic profiles of PSPI, TSPI. Lane 1 – SPI; Lane 2 – Molecular mass standards; Lane 3 – TSPI after 1 h; Lane 4 – TSPI after 2 h; Lane 5 – PSPI after 1 h; Lane 6 – PSPI after 2 h

CSPI, there were continuous molecular mass bands from high molecular masses to low molecular mass.

Thermal studies

In the DSC scans (recorded at a heating rate of 10°C min^{-1}) of soy protein and its various hydrolysed products, one broad endothermic transition was observed below 120°C (Figs 7 and 8). The endotherm was characterized by noting down the temperature of endothermal peak (T_p) position, which was obtained by extrapolation. These results are summarised in Table 1. The T_p values varied in different soy protein products.

SPI, SPC, 7S-RG and 11S-RG fractions showed broad single endothermic transition in the temperature range 35±5 to 135±10°C. SPC had a T_p of 68.0°C, which is higher than SPI and this may be due to the presence of carbohydrates in SPC. The temperatures of maximal deflection (T_p) of endotherm were higher in 7S and 11S in comparison to SPI (Table 1).

The enzymatic hydrolysis of SPI with chymotrypsin (sample CSPI) or urease (USPI) yielded products with higher T_p values. A significant reduction in molecular mass has been observed in CSPI. Low molecular mass polypeptides thus obtained are expected to have higher content of amino and carboxyl end groups which may lead to extensive hydrogen bonding and association with water. Higher temperature would be

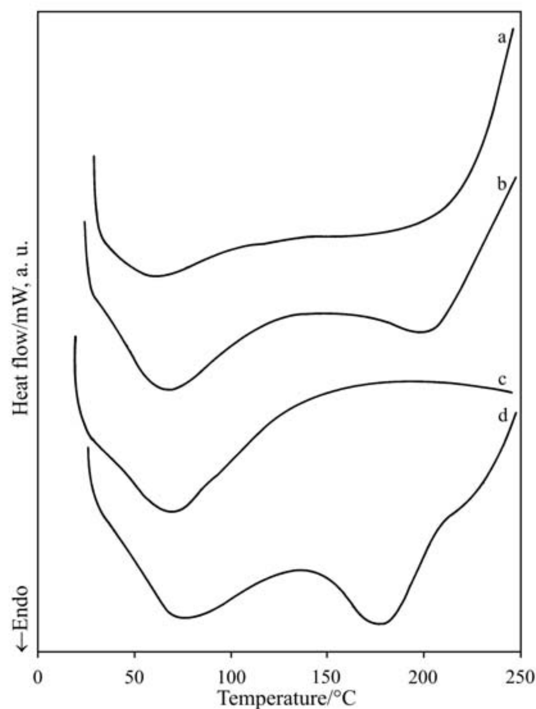

Fig. 7 DSC scan of a – SPI, b – 11S-RG, c – SPC and d – 7S-RG

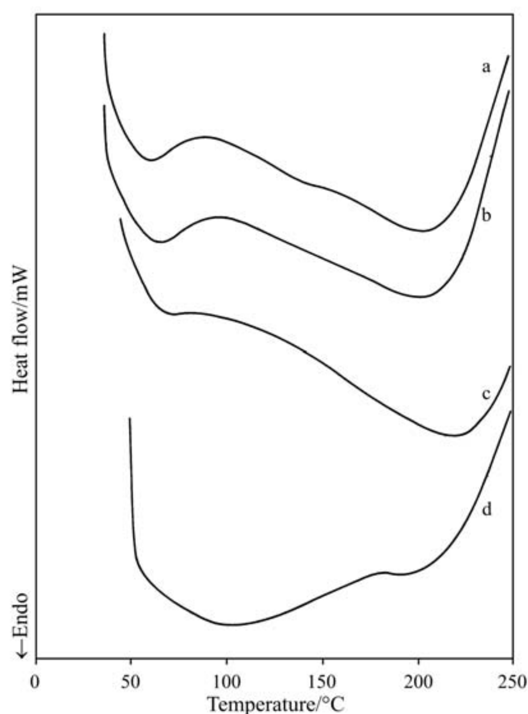

Fig. 8 DSC scan of a – PSPI, b – TSPI, c – USPI and d – CSPI

needed to remove absorbed moisture from such samples. However, when the DSC scans were recorded at a heating rate of 5°C min^{-1} a decrease in T_p values was observed.

Two endothermic transitions region have been reported earlier by Oritz *et al*. [15] for native soy protein isolate. These have been attributed to denaturation of β-conglycinin (79.9°C) and glycinin (95.5°C) fractions of 7S-RG and 11S-RG, respectively. However, these authors did not investigate the possibility of mass loss due to evaporation of absorbed moisture by the proteins. We, therefore, performed the thermogravimetric analysis of these samples in nitrogen atmosphere. Mass losses (5–10%) were observed in the temperature range of 40–132°C (Fig. 9). It may, therefore, be concluded that the endotherm in this temperature range may be due to denaturation of proteins as well as due to loss of absorbed moisture.

A second endothermic transition was also observed in all enzymatically hydrolysed samples. The endothermal peak temperature (T_{p2}) are reported in Table 1. This transition is associated with the degradation of polypeptide chain as indicated by accompanied mass loss in TG in these samples. A sharp exotherm was observed above 200°C in all the samples.

Thermal stability of various samples was studied in nitrogen atmosphere. Multistep mass loss behaviour was observed in all the samples (Fig. 9). The TG traces were characterized by noting down the initial decomposition temperature (IDT), final decomposition temperature (T_f), and associated mass loss (%). The temperature of maxi-

Table 1 Results of the DSC scan of native and enzyme modified SPI (DSC traces recorded at 10 or 5°C min^{-1}

Samples	First endotherm	Second endotherm
	T_p/°C	T_{p2}/°C
SPI	60.1 (53.1)	–
SPC	68.0 (62.1)	–
7S-RG	70.9	179.8
11S-RG	66.4 (52.4)	181.9
*PSPI	59.0	204.0
*TSPI	63.3 (53.8)	203.9 (184.6)
*USPI	69.3	223.0
*CSPI	106.3	197.5

*Enzymatically modified samples
Figures in parenthesis indicate the data obtained at a heating rate of 5°C min^{-1}
T_p, T_{p2}=endothermal peak temperature

mum rate of mass loss was determined from differential thermogravimetric (DTG) trace. These results are summarized in Table 2. In all samples, the first step of mass loss of 7±1% was below 150°C with T_{max} values around 82±10°C except in CSPI where an exceptionally high value of 168°C was observed. This mass loss may be due to absorbed moisture or volatilisation of low molecular mass products of hydrolysis.

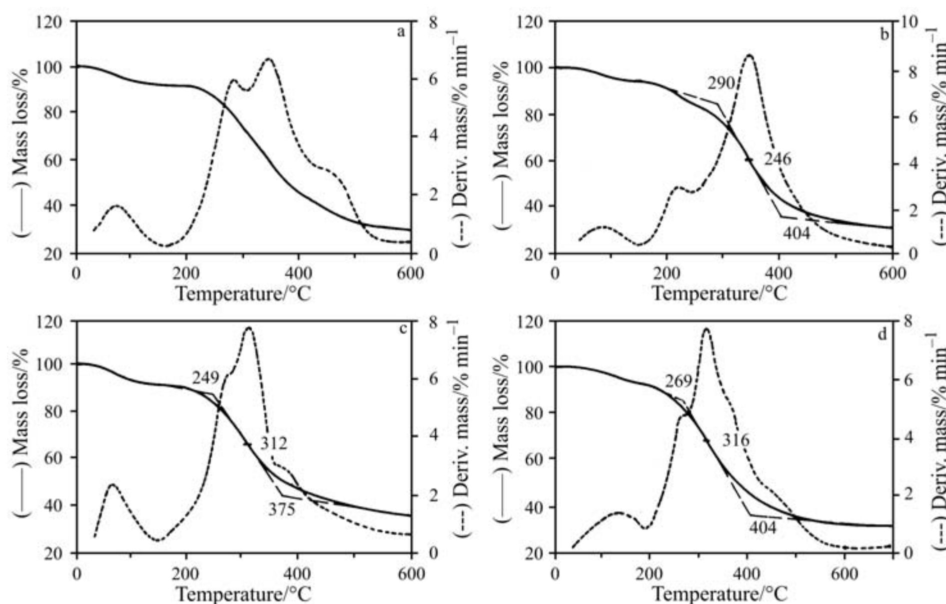

Fig. 9 TG/DTG traces of a – SPI, b – 7S-RG, c – PSPI and d – CSPI

Table 2 Thermal characterization of native and enzyme modified soy protein

Sample	*IDT*/°C	T_{max}/°C	T_f/°C	Mass loss/%	Y_c/%
SPI	31 168 304 462	72 278 340 –	153 303 422 556	7.5 20.7 14.9 26.6	30.3
SPC	31 165	81 344	125 600	5.0 69.9	25.1
7S-RG	37 156 246	81 215 346	137 238 606	5.8 8.1 53.6	32.5
11S-RG	31 172 275	81 254 331	156 268 571	6.7 7.4 55.7	30.2
*TSPI	– 142 383	44 319 –	127 369 545	7.9 37.4 19.9	34.8
*USPI	44 207 436	91 327 –	200 411 545	8.4 41.1 18.0	32.5
*CSPI	39 196 278	168 266 316	186 271 600	7.7 7.4 50.8	34.1
*PSPI	37 150 294 375	69 – 312 –	147 287 365 578	8.2 12.6 21.5 21.6	30.1

*Enzymatically modified samples
IDT – initial decomposition temperature
T_{max} – temperature of maximum rate of mass loss
T_f – final decomposition temperature
Y_c – char yield at 600°C

The second step was observed above 155°C. SPI showed four steps degradation with char yield of 30.3% at 600°C whereas SPC degraded in two steps. The step 2–4 were merged into one. This may be due to complex nature of SPC, which contains carbohydrates along with proteins. The mass loss pattern thus is due to degradation of two different constituents. In DSC scans an exotherm was observed in both the samples (~150°C). The results of DSC and TG studies thus indicate that backbone degradation takes place in SPC and SPI above 150°C. A three-step degradation was observed for 7S-RG and 11S-RG fraction of soy protein suggesting presence of protein fractions of similar nature. This is also supported from gel electrophoresis data (Fig. 4).

Three-stage degradation was also observed in TSPI, USPI and CSPI. A four-step degradation was observed in PSPI. The molecular mass reduction was maximum in sample CSPI but its thermal stability is similar to other enzymatically treated soy protein products. The specificity of this enzyme is also different than

trypsin. This suggests that end group initiated degradation plays no significant role in determining the thermal stability in these polypeptides.

Conclusions

Our studies indicate that enzymatic hydrolysis of soy protein results in significant reduction in molecular mass. The effect of end groups on thermal stability of the hydrolysed products was only marginal. Further studies on characterization of intermediate products obtained after enzymatic and thermal treatment at different temperatures are needed to get insight into the mechanism of the degradation.

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