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Three phase partitioning of starch and its structural consequences

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Abstract

Three phase partitioning (TPP) of starch and its derivatives was carried out by adding *tert*-butanol to the aqueous solution of the polysaccharides in presence of ammonium sulfate. Starch and its modified forms could be recovered in more than 90% yield in the form of an interfacial layer between aqueous and organic phases. Quantitative FT-IR spectral analysis of TPP-treated potato starch revealed a clear change in the IR absorption band of the hydroxyl groups indicating a decrease in hydrogen bonding. TPP-treated starch showed lower biodegradability with wheat germ α-amylase.

Keywords: Biodegradability of starch; Modified starches; Three phase partitioning

1. Introduction

Three phase partitioning (TPP) is a reasonably well-established bioseparation strategy for proteins/enzymes (Dennison & Lovrien, 1997; Sharma & Gupta, 2001a,b). It involves addition of salt (generally ammonium sulfate) and an organic solvent (generally *tert*-butanol) to a crude protein solution. The partially purified protein appears as an insoluble interface between the upper organic solvent phase and lower aqueous phase. TPP treatment of a pure enzyme (Proteinase K) has been shown to result in modification of the structural as well as biological properties of the enzyme (Singh et al., 2001). Recently, we have shown that alginate and chitosan can also be separated by TPP (Sharma & Gupta, 2002; Sharma, Mondal, & Gupta, 2003). Thus, TPP can presumably be extended to other polysaccharides as well. This work describes the TPP of starch from potato and tapioca and some chemically modified starches. It was found that TPP resulted in changes in susceptibility of potato starch to amylolytic hydrolysis.

Modified starches have found numerous biochemical and industrial applications (Fritz, Seidenstucker, Bolz, & Juza, 1996; Ratto, Stenhouse, Auerbach, Mitchell, & Farrell, 1999). TPP treatment may be a powerful tool to produce subtle changes in properties of starch which may be useful for some of these applications.

2. Materials and methods

Starch soluble was purchased from E. Merck Ltd (Mumbai, India). Tapioca starch and chemically modified starches (thin boiling starch, cationic starch, oxidized starch) were kind gifts from Chemtech Marketing (Delhi, India). All other chemicals used were of analytical grade.

2.1. Preparation of starch solution

Starch solution (1%, w/v) was prepared by adding 0.1 g of starch in 10 ml of distilled water and heating the suspension at 70 °C for about 2–3 min till a clear solution was obtained. The solution was cooled to room temperature and diluted appropriately with distilled water to get the desired concentration wherever required.

2.2. Extraction of amylopectin from potato starch

Amylopectin was extracted from potato starch by the method described by Gilbert, Gilbert, and Spragg (1964).

2.3. Estimation of starch and its derivatives by phenol–sulfuric acid test

Phenol solution (25 μl, 80% w/w) was added to varying concentrations of starch (or its derivative) solutions (final volume made to 1.0 ml with distilled water). To the same mixture, 2.5 ml of concentrated sulfuric acid was added

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and kept at 25 °C for 10 min. Absorbance was read at 489 nm (Hirs, 1967).

2.4. Three phase partitioning of starch and its derivatives

Starch solutions (2 ml, various concentrations) were mixed with varying amounts of ammonium sulfate (w/v). Different volumes of *tert*-butanol (v/v) were added to this solution, vortexed and incubated at a suitable temperature for 1 h. Three phases were formed. The upper layer of *tert*-butanol was separated from lower aqueous layer by an interfacial precipitate of the polysaccharide. The precipitated starch in the midlayer was suspended in 2 ml of distilled water and heated at 70 °C for 2–3 min till a clear solution was obtained. It was then cooled to room temperature and dialyzed extensively to remove ammonium sulfate. The amount of the starch in this solution was estimated by phenol–sulfuric acid test. The amount of polysaccharide precipitated was calculated by taking the starting amount of starch as 100%.

2.5. Extraction and purification of wheat germ α-amylase

Wheat germ α-amylase was extracted from wheat germ powder and purified by affinity precipitation as described elsewhere (Sharma, Sharma, & Gupta, 2000).

2.6. Estimation of wheat germ α-amylase activity

Activity of α-amylase was estimated using starch as a substrate (Decker, 1977). One enzyme unit (U) is defined as the amount of enzyme which liberates 1 μmol of reducing sugar (calculated as maltose) per minute at 25 °C and pH 5.6. The reducing end group produced was measured colorimetrically with dinitrosalicylic acid reagent.

2.7. Determination of kinetic parameters

K_m and V_{max} values of wheat germ amylase purified by affinity precipitation, were determined by measurement of enzyme activity with varying concentration of substrate, i.e. starch and TPP-treated starch. Kinetic constants were

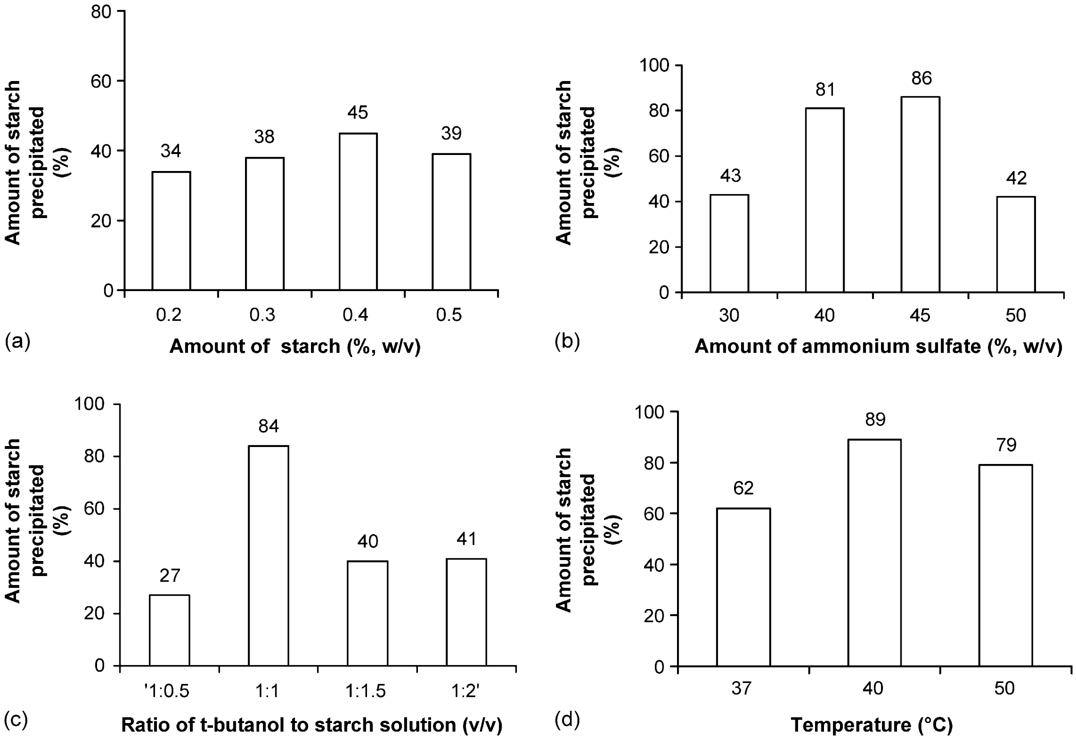

Fig. 1. Optimization of the conditions for precipitation of starch by TPP. (a) Varying concentrations of starch (2 ml) were added to 30% (w/v) ammonium sulfate and mixed with 2 ml of *tert*-butanol. The interfacial precipitate formed was collected after keeping these systems at 40 °C for 1 h. (b) Starch solutions (0.4% w/v, 2 ml) were added to varying amounts of ammonium sulfate (%, w/v), mixed with 2 ml of *tert*-butanol (v/v). The interfacial precipitate formed was collected after keeping the systems at 40 °C for 1 h. (c) Starch solutions (0.4% w/v, 2 ml) were added to 45% (w/v) ammonium sulfate. TPP was carried at 40 °C by varying the ratio of polymer solution to *tert*-butanol (v/v). (d) Starch solutions (0.4% w/v, 2 ml) were added to ammonium sulfate (45%, w/v) and mixed with equal volume of *tert*-butanol. TPP was carried out at different temperatures. In all the above cases, the interfacial precipitate of starch was dissolved in 2 ml distilled water by heating the mixture at 70 °C for 2–3 min. The solution was cooled to room temperature and dialyzed extensively to remove ammonium sulfate. The amount of starch precipitated was estimated by phenol–sulfuric acid test as described in Section 2. Each set of above experiments were carried out in duplicate and the difference in the values within a pair was found to vary within 4%.

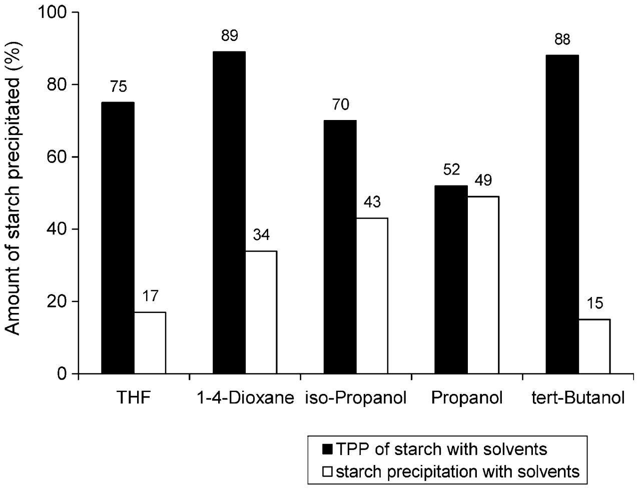

Fig. 2. TPP of potato starch with different organic solvents. Ammonium sulfate (45%, w/v) was added to 2 ml starch solutions (0.4%, w/v), followed by addition of 2 ml of various organic solvents. Three phases were formed after incubating the systems at 40 °C for 1 h. Other solvents tried were dimethyl formamide, dimethyl sulfoxide and *tert*-amyl alcohol. In all these cases, three phase formation did not occur. As a control, 2 ml of the solvents were added to 2 ml of 0.4%, w/v of starch solutions to precipitate it. In all the above cases, the interfacial precipitate of starch was dissolved in 2 ml distilled water by heating the mixture at 70 °C for 2–3 min. The solution was cooled to room temperature and dialyzed extensively to remove ammonium sulfate. The amount of starch precipitated was estimated by phenol–sulfuric acid test as described in Section 2.

calculated using the Leonora software program (Cornish-Bowden, 1995). This software uses the Lineweaver Burke equation to calculate K_m and V_{max}.

2.8. Spectroscopy experiments

FT-IR spectra were recorded on Nicolet-protégé-460 spectrometer. TPP of starch was carried out by adding 45% (w/v) ammonium sulfate to 2 ml of (0.4%, w/v) starch solution mixed with 2 ml of *tert*-butanol. The interfacial precipitate obtained after keeping these solutions at 40 °C for 1 h was dissolved in distilled water by heating it at 70 °C for about 2–3 min, cooled to room temperature and extensively dialyzed. Starch solution (0.4%, w/v, in distilled water) was also dissolved by heating it at 70 °C for same period of time and treated similarly as above. This starch sample was taken as control. The sample as well as the control were lyophilized and their spectra was recorded in the form of KBr pellet.

To prepare the KBr pellets, 1.5 and 3.0 mg of both sample and the control were homogenized with KBr in an agate mortar.

3. Results and discussion

Fig. 1a shows the amount of starch precipitated when its starting concentration was varied. The maximum precipitation (45%) was obtained with 0.4% (w/v) starch solution. This initial trial was made with 30% (w/v) ammonium sulfate and with 1:1 (v/v) ratio of *tert*-butanol to starch solution at 40 °C. These conditions were initially chosen since they have often worked in the case of various proteins (Dennison & Lovrien, 1997; Sharma & Gupta, 2001a,b) and alginate (Sharma & Gupta, 2002). Fig. 1b shows that the precipitation could be increased to 86% when 45% (w/v) of ammonium sulfate was used with 0.4% (w/v) starch solution. Fig. 1c shows that changing the ratio of *tert*-butanol to the aqueous solution did not significantly improve precipitation. Infact, even changing the temperature (Fig. 1d) did not increase the yield of precipitated starch. Thus, 86–89% starch could be precipitated as an interfacial layer with 0.4% (w/v) aqueous solution of starch, 45% (w/v) of ammonium sulfate, 1:1 ratio of organic solvent to the aqueous layer and by carrying out TPP at 40 °C.

Fig. 2 shows that *tert*-butanol was not the only solvent which could be used for TPP. 1,4-Dioxane was equally good and tetrahydrofuran (THF) was a close second under

Table 1
TPP of potato starch with different salts

Salts	Concentration of salts (%, w/v)				
	5	10	20	25	30
NaCl	X	X	X	12% starch precipitated	13% starch precipitated
$CaCl_2$	X	53% starch precipitated	X	X	X
$MgCl_2$	X	X	69% starch precipitated	X	X
NH_4Cl	X	33% starch precipitated	19% starch precipitated	6% starch precipitated	4% starch precipitated
Na_2SO_4	X	X	X	X	X
$MgSO_4$	75% starch precipitated	X	X	X	X

Different salts were added to starch solutions (0.4%, w/v; 2 ml) in varying concentrations. This was followed by the addition of 2 ml of *tert*-butanol. TPP was carried out by incubating these systems at 40 °C for 1 h. The interfacial precipitate of starch was dissolved in 2 ml of distilled water by heating at 70 °C for 2–3 min, dialyzed extensively to remove the salt and estimated as given in Section 2. X, no three phase formation was observed.

Table 2
TPP of different starches and the optimization of conditions for their maximum precipitation

Polysaccharides	Best condition for maximum amount of starch precipitated	Amount of starch precipitated (%)
Potato starch	0.4% w/v, 45% (w/v) ammonium sulfate, 1:1 ratio of starch solution to *tert*-butanol (v/v), 40 °C	89
Tapioca starch	0.4% (w/v), 40% (w/v) ammonium sulfate, 1:1 ratio of starch solution to *tert*-butanol (v/v), 40 °C	98
Cationic starch	0.3% (w/v), 45% (w/v) ammonium sulfate 1:1 ratio of starch solution to *tert*-butanol (v/v), 40 °C	98
Thin boiling starch	0.5% (w/v), 45%, w/v ammonium sulfate 1:1 ratio of starch solution to *tert*-butanol (v/v), 40 °C	96
Oxidized starch	0.5% (w/v), 45% w/v, ammonium (low viscosity) sulfate 1:1 ratio of starch solution to *tert*-butanol (v/v), 40 °C	95
Amylopectin	0.1% (w/v), 45% w/v ammonium sulfate 1:1 ratio of starch solution to *tert*-butanol (v/v), 40 °C	98

the conditions which were optimized with *tert*-butanol. Thus, optimization of conditions with some of these solvents can give greater yield. However, as almost all the studies on TPP of proteins and polysaccharides (Dennison & Lovrien, 1997; Sharma & Gupta, 2002) have been carried out with *tert*-butanol, further work was carried out with *tert*-butanol only. Fig. 2 also shows that TPP as a phenomenon is different from simple precipitation of starch with alcohols and other organic solvents.

Dennison and Lovrien (1997) have discussed the possible mechanism, which might be the basis of TPP of proteins. According to these authors, sulfate works best because of extraordinary hydration and the Hofmeister kosmotropicity. Table 1 shows the results of TPP with different salts of the Hofmeister series. Only magnesium chloride at 20% (w/v) and magnesium sulfate at 5% (w/v) gave good yields of 69 and 75%, respectively. For cations, at least, no correlation could be observed with this series, in the case of potato starch. Among anions, sulfate was better than chloride, which is expected according to the Hofmeister series. However, as pointed out by Dennison and Lovrien, sulfate has multitude of effects.

From practical point of view, it looked prudent to do further work with ammonium sulfate only.

Similarly, the optimization of conditions for TPP was carried out for tapioca starch, other different forms of potato starch and potato amylopectin. These optimum conditions are summarized in Table 2. It is seen that in all the cases, more than 90% polysaccharide could be obtained as a precipitate by TPP. Thus, TPP may be an attractive choice for extraction/purification of starch and its derivatives.

TPP is known to alter the tertiary structure of Proteinase K (Singh et al., 2001). In other cases as well, the turn over

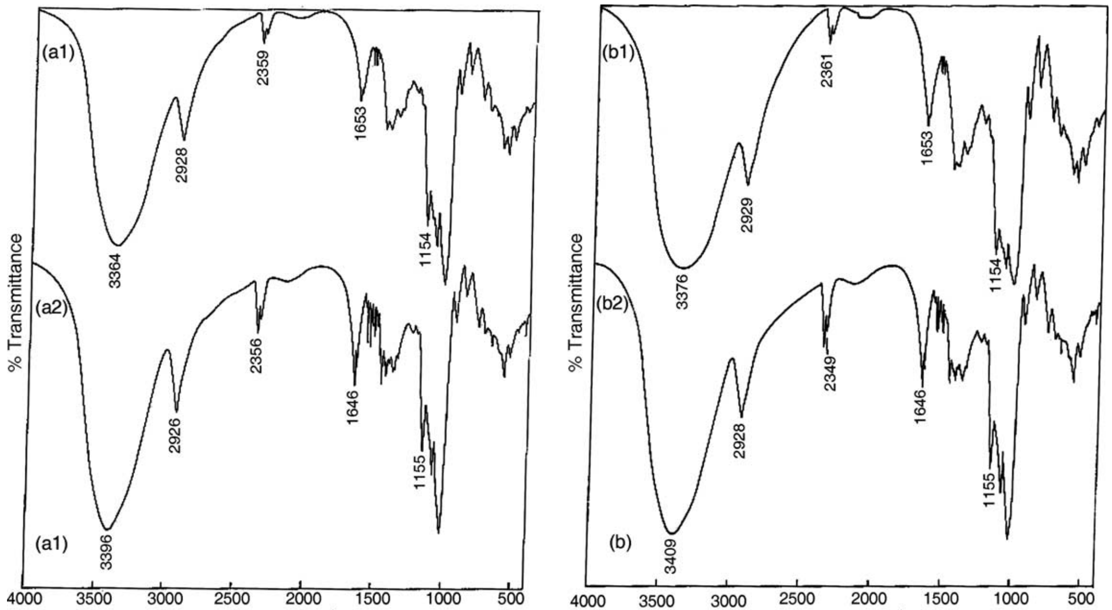

Fig. 3. Fourier-transform infrared (FT-IR) spectra of starch and TPP-treated starch. FT-IR spectra of starch and TPP-treated starch was recorded on Nicolet-protégé-460 spectrometer as described in Section 2. FT-IR spectra of 1.5 mg starch (a1) and 1.5 mg TPP-treated starch (a2). FT-IR spectra of 3.0 mg starch (b1) and 3.0 mg TPP-treated starch (b2).

Table 3
Determination of kinetic parameters of wheat germ α-amylase using starch and TPP-treated starch as substrate

Kinetic parameters	Starch	TPP-1 starch	TPP-2 starch	TPP-3 starch
K_m (mg/ml)	14.47	22.13	10.26	31.54
V_{max} (μmol/min nmol enzyme-1)	0.636	0.552	0.295	0.616
V_{max}/K_m	0.044	0.025	0.028	0.020

TPP-1, the first TPP of starch. This was carried out by adding 45% w/v ammonium sulfate and *tert*-butanol in a ratio of 1:1 (v/v) to 0.4% w/v starch solution. Three phases formed were collected after incubating the solutions at 40 °C for 1 h. Eighty eight percent of starch was precipitated as an interfacial layer. TPP-2, the starch obtained from TPP-1 (as 88%) was dialyzed and subjected to a second TPP under the same conditions as described above. Eighty five percent starch precipitated as an interfacial layer. TPP-3, starch precipitated in TPP-2 was dialyzed and subjected to a third TPP under the same conditions as described above. Seventy five percent starch precipitated as an interfacial layer. The starch precipitated as TPP-1, TPP-2 and TPP-3 were dialyzed, lyophilized and subjected to amylolytic hydrolysis using wheat germ α-amylase.

number of enzymes is reported to be significantly enhanced (Dennison & Lovrien, 1997). Thus, it was considered worthwhile to probe the structural consequences of carrying out TPP with potato starch.

FT-IR spectra of starch and TPP-treated potato starch is shown in Fig. 3. The FT-IR of untreated starch agrees well with recently reported spectra (Fang, Fowler, Tomkinson, & Hill, 2002).

Both the spectra were recorded at two different concentrations of the polysaccharides (1.5 and 3.0 mg) and it was found that the hydroxyl peak in case of TPP-treated starch shifted to higher wavenumber by about 30 cm^{-1}. As the difference between the position of hydroxyl peak of starch and TPP-treated starch is same at both concentrations of the polysaccharide, this indicated that there may be decrease in intra-molecular hydrogen bonding (i.e. the hydrogen bonding between monomers of the same starch molecule) rather than affecting inter-molecular hydrogen bonding (i.e. hydrogen bonding between different starch molecules).

Table 3 shows the susceptibility of starch (after repeated TPP) to amylolytic action of wheat germ α-amylase. This susceptibility has been evaluated in terms of how good a substrate starch was before and after various TPP treatments. V_{max}/K_m is generally taken as an indication of this (Segel, 1976). No clear trend could be observed since TPP-2 looked to be a better substrate than TPP-1 and TPP-3. However, in general, TPP treatment resulted in a molecule, which was a poor substrate as compared to untreated starch. Starch is being used as a biodegradable material (Aburto et al., 1999). One disadvantage is that amylases are ubiquitous enzymes and starch is readily hydrolyzed. Thus, moderation of its hydrolysis by TPP should further its potential as a biodegradable material of optimum degradability.

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