SUPERMOLECULAR STRUCTURE AND PROPERTIES OF GLYCEROL MONOSTEARATE-AMYLOSE COMPLEXES

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Himalee D. Seneviratne

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HIMALEE D. SENEVIRATNE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TO MY BELOVED PARENTS

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TABLE OF CONTENTS

ACKNOWLEDGMENTS.....ii

TABLE OF CONTENTS.....iii

LIST OF TABLE.....vi

LIST OF FIGURES.....vii

ABSTRACT.....x

FOREWORD.....xiii

INTRODUCTION.....1

REVIEW OF LITERATURE.....4

Molecular Structure of Starch.....4

Structure and Properties of Amylose.....5

Structure and Properties of Amylopectin.....12

Starch Granular Structure.....15

Starch-Lipid Interactions.....18

Functionality Aspect of Amylose-Lipid Interactions.....21

Structure of Amylose-V Complexes.....23

Solvent Effects on Macromolecules.....36

Salt Effects on Stability of Macromolecules.....37

Effects of Sugars on Stability of Macromolecules.....41

Enzyme Digestion of Starch.....42

Enzymes Involved in Hydrolysis.....43

Factors Effecting Enzyme Availability of Starch.....45

MANUSCRIPT 1.....48

Introduction.....49

Materials and Methods.....52

Preparation of the complex.....52

Acid Hydrolysis.....53

X-ray Diffractometry.....54

Differential Scanning Calorimetry.....54

Results and Discussion.....55

Annealing Effects and Structural Considerations.....55

Solvent Effects on Conformational Stability 67

MANUSCRIPT II.....92

Introduction.....93

Materials and Methods.....97

Preparation of Glycerol Monostearate-Amylose Complex...97

X-ray Diffraction Analysis.....98

Differential Scanning Calorimetry.....98

Results.....99

Discussion.....117

MANUSCRIPT III.....127

Introduction.....128

Materials and Methods.....131

Preparation of the Complexes.....132

α-amylase Digestion.....132

X-ray Diffractometry.....133

Differential Scanning Calorimetry.....134

Turbidity Measurements.....134

Debranching of Waxy Maize Starch with Pullulanase.....134

Gel Chromatography.....135

^{13}C CP/MAS NMR.....135

Results and Discussion.....136

Structural Considerations.....136

α-amylolysis of Complex.....143

Physical Properties of Enzyme-resistant Fractions.....154

Acknowledgments.....161

SUMMARY AND CONCLUSIONS.....162

CONTRIBUTIONS TO KNOWLEDGE.....166

LITERATURE CITED.....168

LIST OF TABLES

TABLE 1. Thermal and chemical properties of amylodextrin residues after acid-etching of glycerol monostearate -amylose complex (form I and II) in heterogeneous reaction mixture.....64

TABLE 2. Transition enthalpies of glycerol monostearate-amylose complexes (form I and II) as a function of molar concentration of various sodium salts.....105

TABLE 3. Time-dependent effects on the thermal properties of glycerol monostearate-amylose complexes (form I and II) in the presence of NaSCN.....106

TABLE 4. Transition enthalpies of glycerol monostearate-amylose complexes (form I and II) as a function of molar concentration of various salts with chloride as common counterion.....115

TABLE 5. Extent of hydrolysis of glycerol monostearate-amylose complex (form IIa) by α-amylase in a heterogeneous reaction mixture.....147

TABLE 6. DSC characteristics of wheat starch granules and glycerol monostearate-amylose complexes after hydrolysis with *Bacillus subtilis* α-amylase.....155

TABLE 7. DSC characteristics of wheat starch granules and glycerol monostearate-amylose complex after hydrolysis with hog pacreas α-amylase.....156

LIST OF FIGURES

Figure 1 . Chemical structure of amylose.....7

Figure 2 . Comparison of unit cells and helix packing of A (left) and B (right) amylose.....9

Figure 3 . X-ray diffraction tracings from different starches.....11

Figure 4 . Chemical structure of amylopectin.....14

Figure 5 . Proposed cluster model for the molecular structure of amylopectin.....17

Figure 6 . Schematic representation of the arrangement of amylopectin molecules within a growth ring of a granule.....20

Figure 7 . Representation of the amylose-lipid complex.....27

Figure 8 . DSC thermal curves of amylose-monoglyceride complexes crystallized at various temperatures.....30

Figure 9 . X-ray diffraction diagrams of wet amylose-monoglyceride complexes obtained at various crystallization temperatures.....33

Figure 10. Generalized mechanism for amylose lipid complex formation from dilute solutions.....35

Figure 11. Effect of annealing temperatures (2 hr at the specified temperature) on the structure of forms I and II.....58

Figure 12. X-ray diffraction diagrams: forms I and II.....60

Figure 13. Chromatographic profiles of resistant amylodextrin fragments after acid hydrolysis of the complex on a Sephacryl S-200 column.....66

Figure 14. DSC thermal curves of structural forms I and II of the complex in sucrose solutions of varying molar concentrations.....70

Figure 15. DSC thermal curves of structural forms I and II of the complex in Na_2SO_4 solutions of varying molar concentrations.....72

Figure 16. DSC thermal curves of structural forms I and II of the complex in urea solutions of varying molar concentrations.....76

Figure 17. DSC thermal curves of structural forms I and II of the complex in guanidine hydrochloride solutions of varying molar concentrations.....78

Figure 18. Transition enthalpies of structural forms I and II of the complex with molar concentration of guanidine hydrochloride and urea.....81

Figure 19. X-ray diffraction patterns of dehydrated freeze-dried samples and DSC thermal curves of forms I, IIa and IIb....83

Figure 20. DSC thermal curves of structural forms I, IIa and IIb of the complex in cesium chloride solutions of varying molar concentrations.....86

Figure 21. X-ray diffraction diagrams of wet glycerol monostearate -amylose complexes of form I, IIa and form IIb in CsCl solutions of varying concentrations.....88

Figure 22. Transition enthalpies of structural forms I, IIa and IIb of glycerol monostearate-amylose complexes with molar concentration of CsCl.....91

Figure 23. DSC thermal curves and X-ray diffraction patterns of wet glycerol monostearate-amylose complex superstructures.....101

Figure 24. Changes in the melting temperature of glycerol monostearate-amylose complexes (form I and II) as a function of molar concentration of various sodium salts.....103

Figure 25. DSC thermal curves of structural forms I and II in NaI solutions of various molar concentrations.....109

Figure 26. Changes in the melting temperature of glycerol monostearate-amylose complexes, form I and II, as a function of molar concentration of various salts with chloride as common counterion.....111

Figure 27. DSC thermal curves of glycerol monostearate-amylose complex (form I) at complex to sugar to water weight ratios of 2.0:1.6:6.4 and 1.0:2.0:2.0.....114

Figure 28. Transition temperature of forms I and II as a function of 100/MW of cosolvent for 3-component mixtures of glycerol monostearate-amylose : sugar : water (1.0:2.0:2.0 parts by weight).....126

Figure 29. DSC thermal curves, X-ray diffraction patterns and ^{13}C CP/MAS NMR spectra of glycerol monostearate-amylose complexes (forms I, IIa and IIb).....138

Figure 30. Time dependent changes in turbidity of forms I, IIa and IIb of glycerol monostearate-amylose complex suspensions (0.37 mg/ml).....142

Figure 31. Enzyme hydrolysis of wheat starch and forms I, IIa and IIb (0.5g solids/60ml buffer, 3.3IU/100mg substrate) of glycerol monostearate-amylose complexes by *B. subtilis* and hog pancreas α-amylases.....145

Figure 32. Chromatography on Sepharose CL-6B of native and complex residues after hydrolysis with *B. subtilis* and hog pancreas α-amylases.....150

Figure 33. X-ray diffraction patterns of wet glycerol monostearate-amylose complexes of native and residues of *B. subtilis* and hog pancreas α-amylase-treated complexes....153

Figure 34. DSC thermal curves of solid residues of Form I following hydrolysis with *B. subtilis* α-amylase for a specific reaction time.....159

ABSTRACT

Calorimetry of glycerol monostearate-amylose complexes reveals the presence of at least two thermally distinct metastable forms, which implies a definite connection between thermal properties and supermolecular organization of these materials. On the basis of X-ray-diffraction, calorimetric, and structural-analysis data, it is postulated that form I (Tm 99.4^{0}C), obtained at lower crystallization temperatures, has a uniformly distributed partial order where no separate crystallites can be identified. Such structure would have an internal energy and entropy between those of an amorphous melt and a structure of discrete crystallites, such as form II (Tm 116.7^{0}C). Conversion of form I to form II is carried out by isothermal annealing at temperatures above its melting point. The conformational responses of the complex (form I and II) in various stabilizing (Na_2SO_4, sucrose, CsCl) and destabilizing (urea, guanidine hydrochloride) environments further suggest that interconversion between the various forms can be explained by considering that structural order at two levels are affected: association-dissociation of aggregated helices and helix-coil transitions; at high concentrations, CsCl caused disruption of crystallites without altering the conformation of individual helices. The transition enthalpy of the complex shows very little change with increases in the long-range order of the supermolecular structure (X-ray diffraction data) and is interpreted to reflect mainly contributions from conformational disordering of helices.

Thermally induced order-disorder transitions of the two distinct structural forms of glycerol monostearate-amylose complex (form I and form II) have been investigated by differential scanning calorimetry in various solvent environments. For neutral salts, the effectiveness of anions and cations in stabilizing or destabilizing the ordered chain domains of the complex followed, in general, the classical Hofmeister series. Thus, with Na^+ as the sole counterion, the Tm of the transition (at <0.1M) increased in the order of SCN^-< I^-< NO_3^-< F^-< Cl^-< CH_3COO^-< SO_4^{2-}. With Cl^- as common anion, the ranking of cations was NH_4^+< K^+< Na^+< Li^+≤ Ca^{2+}≤ Mg^{2+}. Interestingly, ranking of certain ions (e.g. CH_3COO^-) differed between the two forms of the complex, particularly at high electrolyte concentrations. This implies that some solutes can act differently at various levels of supermolecular structure. Glucose and maltooligosaccharides were effective stabilizers and resulted in non-equilibrium phase transition behaviour for the metastable superstructures of the complex (melting with reorganization during heating). These effects were proportional to the molecular weight and concentration of the small carbohydrate solute.

Helical inclusion complexes of amylose with glycerol monostearate, varying in their supermolecular structure (as assessed by X-ray, differential scanning calorimetry (DSC), and cross polarization/ magic angle spinning ^{13}C NMR), were subjected to α-amylolysis in the solid state using the *B. subtilis* and porcine pancreatic α-amylases. The rate and the extent of hydrolysis of complexes were inversely related to the degree of organization of helices into larger domains of ordered chains in the aggregated structure; complexes with greater crystallinity were more resistant to enzymic degradation. However, even crystalline forms of

complexes can be fully degraded under prolonged digestion time and high enzyme levels. Gel permeation chromatography of the enzyme-resistant fractions also revealed distributions of longer chains for the more perfected complex superstructures. The DSC and chromatography data on enzyme resistant fractions indicated that the *B. subtilis* α-amylase exerts a more uniform hydrolytic action on the complexes, compared to wheat starch granules. Although enzymic breakdown of amorphous amylose chains (complexed and non-complexed chain segments) in the complex structure did not substantially alter the crystallinity of enzyme-resistant fractions, it reduced their thermal stability (lower transition enthalpy and dissociation temperature).

FOREWORD

This thesis is written in manuscript style. The first manuscript has been accepted by *Carbohydrate Polymers*. The second and third manuscripts have been submitted to *Carbohydrate Research* and *Journal of Cereal Science*, respectively.

INTRODUCTION

It is well known that lipids or surfactants act as texture modifiers when added to starch-containing foods. For example, emulsifiers and lipids are incorporated in cooked and baked products because of their ability to elevate the gelatinization temperature, maintain structural integrity of cereal kernels and retard firming and retrogradation of starch. Although the mechanism by which these effects are brought about is not fully understood, the formation of helical inclusion complexes of amylose with lipids is generally regarded as the main reason for such effects. In the presence of lipids, conformational ordering of amylose (coil——>helix) is induced, which leads to nucleation and organization of helices into partially crystalline chain aggregates, known as V-complexes. Understanding of the structural features of V-amylose has advanced mainly from X-ray diffraction studies (Mikus *et al.* 1946; Rundle and Edwards 1943). However, despite the well known unit cell dimensions and the number of residues per helical turn of these complexes a complete description of the supermolecular structure of V-complexes is a difficult task, since helices can exist in various states of aggregation depending on the thermomechanical history of the product.

Although much work has been carried out on the amylose-lipid interactions, very little attention has been paid to consideration of supermolecular organization of the complex. In fact, in a number of

calorimetric studies (Stute and Konieczny-Janda 1983; Biliaderis *et al.* 1985; Eliasson 1988; Raphaelides and Karakalas 1988), multiple melting transitions have been reported, thus implying a definite connection between thermal properties and supermolecular organization. However, no attempt has been made to provide a possible morphological model for the description of the solid state structure that can account for such thermal responses. The main reason for the lack of progress in this area has been the difficulty in characterizing and quantifying such morphological features as size and shape of the crystallites, relative extent of folding adjacent to the ordered regions, as well as the nature of the amorphous material.

In recent studies on the effects of crystallization temperature on the structure of glycerol monostearate-amylose complexes in solution or upon extrusion of cereal flours (Biliaderis and Galloway 1989; Galloway *et al.* 1989), two thermally distinct forms (Form I and II) were identified. A working structural model for these metastable forms was also proposed. Form I (low Tm) was assumed to be obtained under conditions favouring rapid nucleation and was morphologically described as aggregated helices having little crystallographic register with one another. In contrast, form II (high Tm) appeared to have the classical structure of partially crystalline polymers with well defined crystallites. Thus, it is important to know more about the supermolecular structure and the factors affecting the stability, interconversion and digestibility of these forms, since the supermolecular structure is the functional state of V-amylose complexes in starch based food products.

In this regard, the objectives of this study were:

i) To obtain additional evidence to the hypothesis that form I and II of the glycerol monostearate-amylose complexes are two distinct states;

ii) To examine the stability of these forms in various solvent environments;

iii) To investigate the effects of supermolecular organization of the complex on its resistance toward enzyme (α-amylase) hydrolysis and obtain further insights on the morphology of the solid state.

REVIEW OF LITERATURE

Molecular Structure of Starch

Starch, one of nature's energy reserves, is a widely distributed carbohydrate material, synthesized and stored in various plant tissues and in a variety of granular forms. The molecular structure of starch has been extensively studied (Greenwood 1979; Hood 1982: Daniel 1984; Zobel 1988) and has been identified as an α-D glucan that has two structurally distinct components; linear amylose and branched amylopectin. A third intermediate fraction that seems to be a less branched amylopectin or a slightly branched amylose has also been identified for some starches (Banks *et al.* 1971). In all starch molecules, glucose is the basic building block and the chair ($^4C_1$) form of α-D glucose is the only conformer of the polymeric chains (Whistler and Daniel 1984).

Most starches, including tuber and cereal starches, contain 15-25% amylose. However, certain varieties of waxy cereal starches are virtually free of amylose, while some genetically selected varieties of cereals and legumes have increased amylose contents in the range of 50-70%. In addition to these anhydroglucose polymers, starch granules also contain small amounts of non-carbohydrate components, particularly lipids, proteins, phosphorus, pentosan and ash that can effect the functional behaviour of starch in various applications (Lowy *et al.* 1981).

Structure and Properties of Amylose

The linear fraction of starch, amylose, is usually identified by its affinity for iodine complexation in aqueous solutions. This fraction binds iodine approximately 20% of its own weight (Banks and Greenwood 1975). Amylose is polydispersed with weight average molecular weight usually ranging from 100,000 to 1,000,000 g/mol, depending on the botanical source of the starch and method of extraction. The anhydroglucose units in amylose are linked by α-(1-4) bonds, as shown in figure 1. Amylose can be found in several helical structural polymorphs which include double helices, collapsed helices and extended helices depending on the environment present. For the double helical form, two polymorphic structures have been suggested, A and B (Sarko and Wu 1978). In both cases six glucose residues complete one turn taking 21Å along the axis (Sarko and Wu 1978). Both chains of the double helices are right handed although some recent electron diffraction studies support a left handed helical conformation (Hinrichs *et al* 1987).

As shown in figure 2, the helices in the B starch form columns that cluster about an open space that is filled with 36 molecules of water. In contrast, the proposed A structure (figure 2) is more compact with a double helix filling the central cavity. Thus, an B to A transition could occur by a simple shifting of a helix into the channel occupied by water (Sarko and Wu 1978). However, water is not required to maintain the packing arrangement of molecular chains of the B structure (Kainuma and French 1972). The X-ray diffraction patterns from different starches are presented in figure 3. The starch classification shown here is one of the

Figure 1 . Chemical structure of amylose.

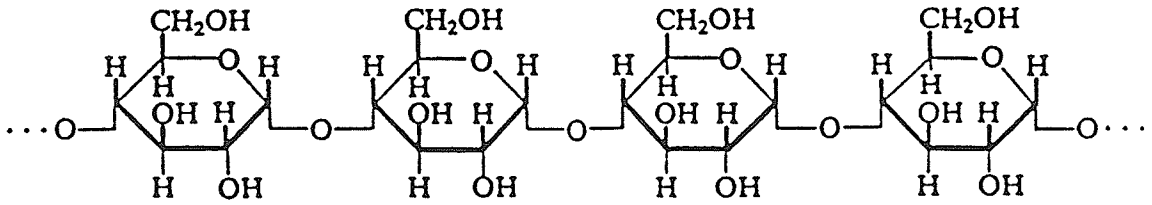


Figure 2 . Comparison of unit cells and helix packing of A and B amylose (Sarko and Wu 1978).

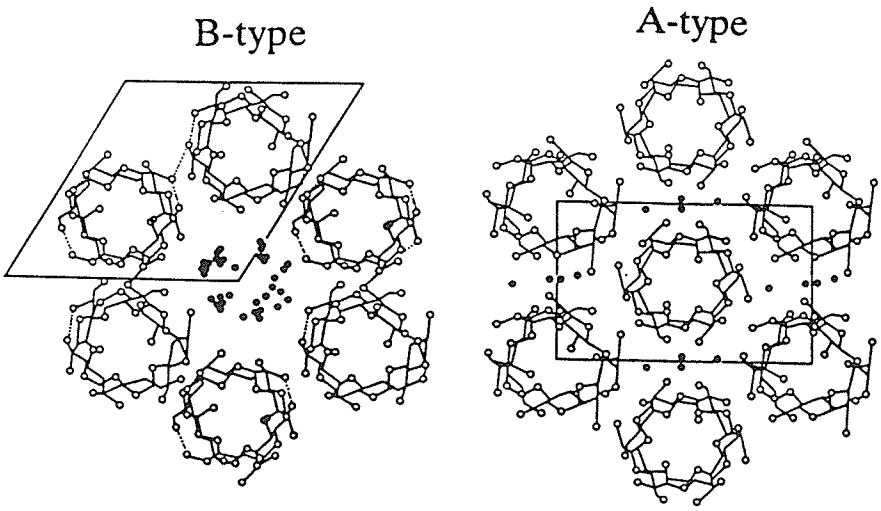
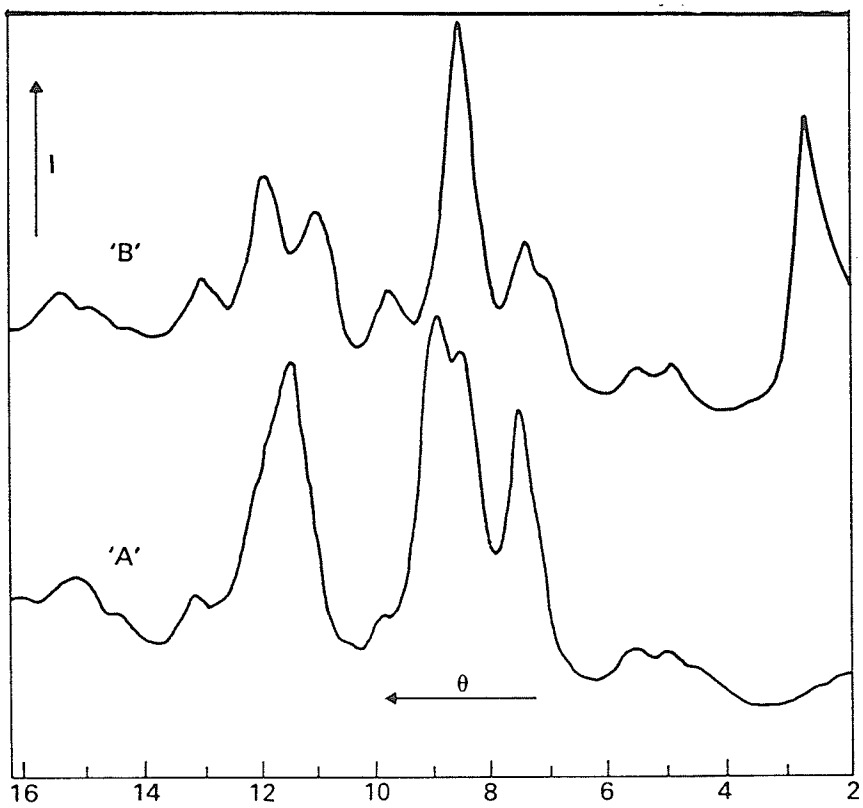

B-type
A-type

Figure 3 . X-ray diffraction tracings from different starches (Zobel 1988b).


I
'B'
'A'
θ
16
14
12
10
8
6
4
2

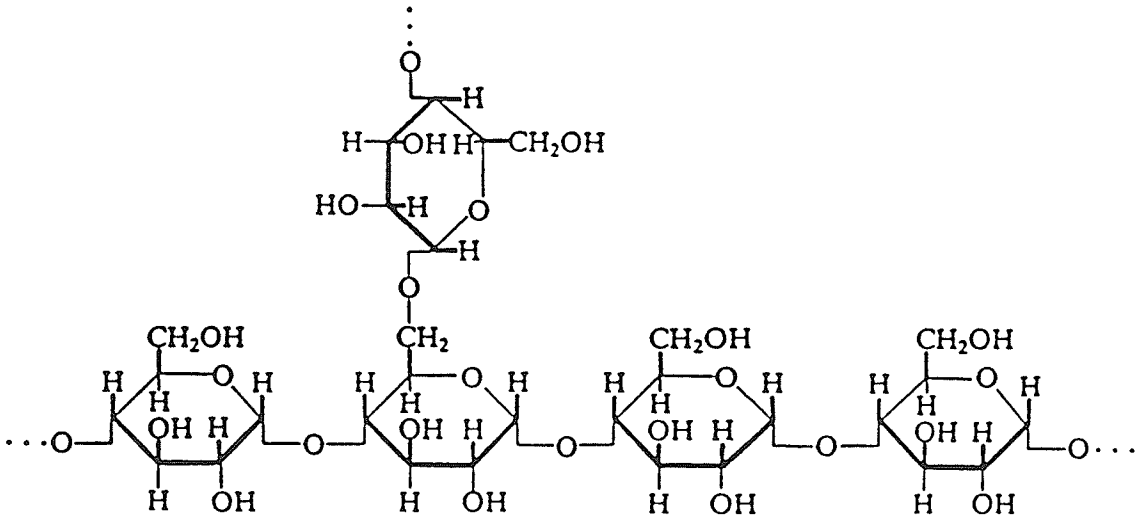
few ways that starch types can be successfully grouped according to a physical property. Generally, cereal starches give A patterns, tubers yield B patterns and certain root and seed starches give C patterns which are thought as mixture of A and B.

In the double helix, each repeat unit in one strand has a partner unit on the other. However of the many juxtaroposed glucose, only the central units are close enough so that intermolecular hydrogen bonds between O-2——O-3 can exist. Between the O-6 hydroxyls there is only limited direct short intermolecular, O-6——O-6 hydrogen bonding of 2.86Å. The hydrogen bonding between O-2——O-3 on one side of the helix and between O-6 hydroxyl on the other contributes significantly, if not primarily, to the stabilization of the helices (Hinrichs *et al*. 1987).

Structure and Properties of Amylopectin

Amylopectin, the branched starch component, has α-(1-4), and α-(1-6) linkages (figure 4). The molecular weight is in the order of 10^7-10^8, and is one of the largest molecules in nature (Hood 1982). Because of its dominance in the starch granule (ie 54-100%), the structure and properties of amylopectin have been widely studied (Robin *et al*. 1975). The most popular model given for its structure is the "cluster" model, which contains three different types of linear chains, designated as A, B and C chains. The A to B ratios can range from 4:1 to 9:1, depending on the amylopectin source. The A and B chains are of different lengths and their degree of polymerization (DP) can vary. In general, the linear A and B chains in most amylopectins have average DP values of 15 and 45,

Figure 4 . Chemical structure of amylopectin.

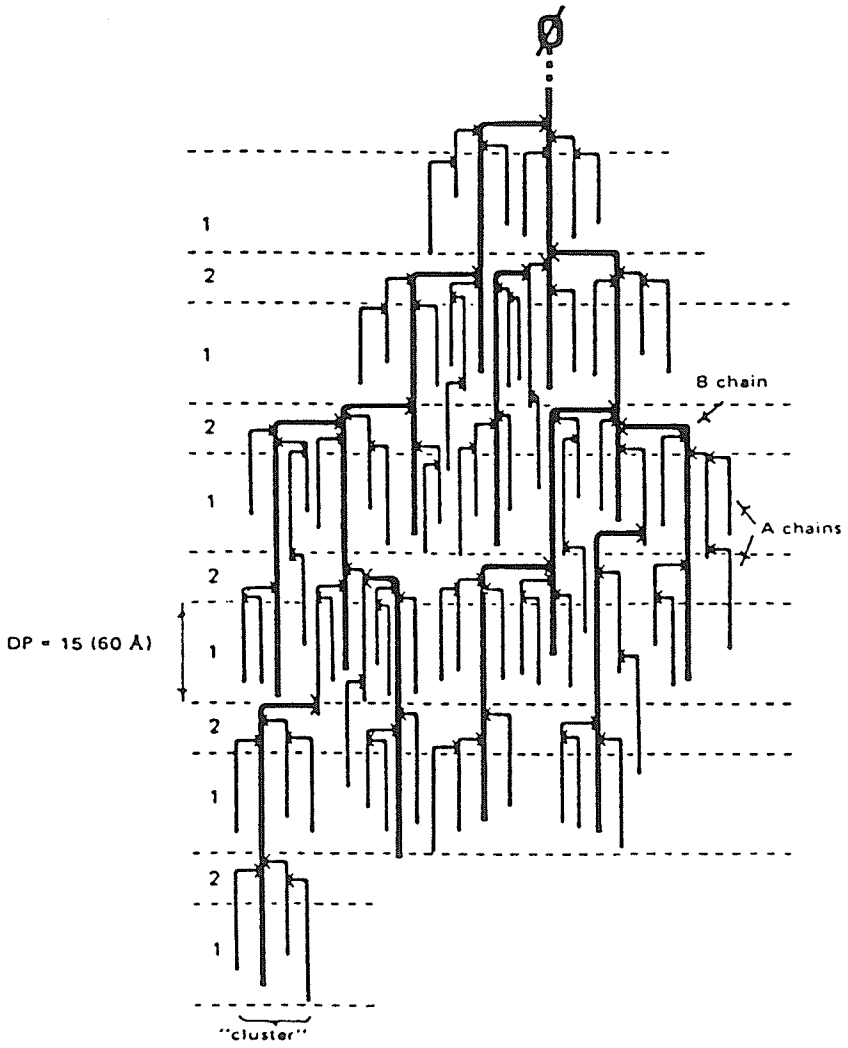


respectively; figure 5 illustrates the A, B and C designations that have been given to chain segments and the clustering of the outer molecular chains (French 1984; Robin *et al.* 1974; Hood and Mercier 1978; Zobel 1988). The A chains are joined to the remainder of the molecule with a single 1→6 bond, B chains are also joined though a 1→6 bond but may carry one or more A and/or B chains on the primary hydroxyl groups. The single C-chain carries the sole reducing group. The linear portions of the short and long chains form double helices with one another and/or with the amylose molecules, and are responsible for the crystalline regions within the starch granule. Unlike amylose, amylopectin does not give the characteristic starch-iodine (amylose-iodine) blue colour, instead a purple and sometimes reddish-brown colour is obtained depending on its source.

Starch Granular Structure

In nature, the amylose and amylopectin polymers are organized into starch granules. Although understanding of the molecular structure of starch is significantly advanced, the knowledge of how these molecules are arranged within the granule is still unclear. In its granular form, starch is semicrystalline, water insoluble and dense. Starch granules vary in size (from 2-100μm) and shape (round, oval or irregular) depending on the plant source. In spite of these varietal differences in the granule size and shape, all starches appear to have similar fine, submicroscopic structures (Lineback 1984).

Figure 5 . Proposed cluster model for the molecular structure of amylopectin: 1= crystalline area; 2= amorphous area; θ= reducing end group; →= α-(1-6) branching points; -= α-(1-4) glucan (Robin *et al.* 1975).


1
2
1
2
1
2
1
2
1
2
1
B chain
A chains
DP = 15 (60 Å)
"cluster"

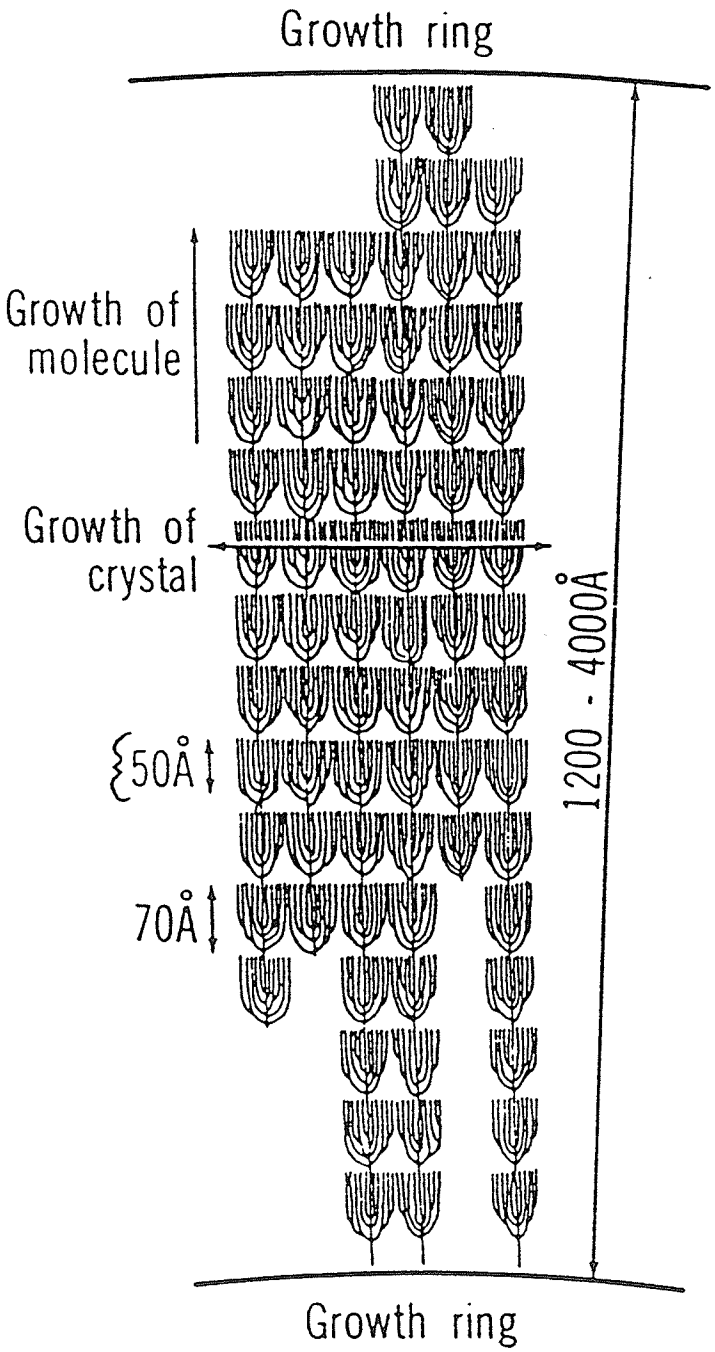
The study of starch granule structure has advanced via a variety of techniques. Optical (light) microscopy and scanning electron microscopy have provided information on some structural features of the granule such as surface morphology and granular architecture, while the fine structural details were revealed by X-ray diffraction, enzymic methods and electron diffraction studies. According to these studies, the starch molecules are radially oriented within the granule (figure 6) (Hood 1982).

Out of the non-carbohydrate components present in the granule, lipids constitute the main minor component. These lipids have been divided into two types; one which can be easily extracted using fat solvents (ie, petroleum ether) is defined as extraneous lipids and the other which cannot be easily extracted, making the internal granular lipids (Morrison 1979, 1981).

Starch-Lipid Interactions

The granular lipids and/or added lipids or surfactants give rise to starch lipid interactions which play an important role in changing the texture and mechanical properties of starch based food systems. Some of the properties to which amylose-lipid complexes contribute are: (1) elevate the gelatinization temperature of granular starches; (2) maintain structural integrity of cereal kernel (i.e. parboiled rice); (3) decrease stickiness and solubility; (4) reduce retrogradation rate (staling) in baked products; (5) improve freeze-thaw stability; and (6) fix and protect volatiles losses.

Figure 6 .Schematic representation of the arrangement of amylopectin molecules within a growth ring of a granule (French 1982).


Growth ring
Growth of molecule
Growth of crystal
50Å
70Å
1200 - 4000Å
Growth ring

Functionality aspects of amylose-lipid interactions

Considerable interest has been forcussed on the effects of lipid addition to starch systems due to the important functionalities it imparts on food products. Gray and Schoch (1962) observed that the addition of a series of saturated fatty acids and monoglycerides on corn and potato starch causes reduction in granular swelling and starch solubilization when heated up to 95^{0}C. Furthermore, the solubility and swelling power of isolated, nondefatted (NDFS), defatted (DFS) and re-embraced (RES) rice starches with fatty acids (i.e. 0.5 and 1.0% palmitic acid) indicated that the DFS released a high percentage of soluble material compared to RES and non-defatted starch. According to iodine staining of the soluble material, more than 70% was amylose (Ohashi *et al.* 1980; Kim and Hill 1985).

Mercier (1980) and Mercier *et al.* (1979; 1980) found that twin screw extrusion cooking of corn, potato and manioc starches in the presence of native and added lipids caused the formation of amylose-lipid complexes which decreased the susceptibility of starches to α-amylase digestion. One of their studies dealt with the effects of adding various lipids to manioc starch. These lipids included saturated and unsaturated fatty acids (C_{12} to $C_{18:2}$) and two surfactant molecules (glycerol monosterate (GMS) and stearoyl lactylate (SSL)). All lipid additions caused formation of V-amylose complexes, with the exception of triglycerides. These complexes decreased the water soluble starch relative to extruded starch without lipid addition. The water solubility also decreased with an increase in the fatty acid chain length. They further studied the freeze-thaw

stability of the water-soluble carbohydrates from the extruded products. At -20^{0}C after five freeze-thaw cycles, the amount of retrograded material decreased with the addition of fatty acids. This change was also related to the chain length of the fatty acid. Thus, it was concluded by these researchers that adding fatty acids or monoglycerides during extrusion can produce a modified starch having technological properties such as low solubility, reduced stickiness and increased freeze-thaw stability. These starches were found competitive for consumer acceptance with chemically modified starch. The pasting temperature and vicosity profiles of corn and wheat starch pastes indicated that the addition of fatty acids and surfactants eliminated the pasting peak, reduced set back viscosity and decreased pasting temperature (Takahasi and Seib 1988; Medcalf *et al.* 1968).

Potato starch gelatinization during precooking or steam cooking is usually accompanied by leaching of straight-chain low molecular weight amylose. This gives the mashed potato a gluey or sticky texture. Several processing steps are applied to reduce this effect and one of this is the addition of monoglycerides. Hoover and Hadziyev (1981) studied the influence of monoglycerides on the extent of amylose leaching and on heated granular starch integrity using scanning electron microscopy (SEM). They observed that at temperatures above starch gelatinization temperature, the granules are surrounded by a continuous filamentous network of amylose molecules leached out of the granule. In the presence of 0.25% monopalmitin, the monoglyceride prevented amylose leaching from heated starch granule, thus preserving the granular integrity above the gelatinization temperature. Thus, it was suggested that at levels of 0.25%

of added monoglycerides, the monoglycerides diffused into the granule and insolubilized the amylose within the granule.

Starch gels, prepared by cooling concentrated aqueous dispersions of gelatinized starches, undergo changes in their rheological properties (firmness), crystallinity and water holding capacity. These processes, collectively described by the term retrogradation have major influence on the texture and acceptability of starch containing foods. For example staling of bread and other baked goods have been attributed to this phenomenon (Schoch and French 1947). Although the actual mechanism, particularly at a molecular level, is not clear the addition of lipids considerably reduces the rate of retrogradation in both model and actual composite starch systems (Eliasson 1983; Russell 1983; Evans 1986; Eliasson *et al* 1988).

Structure of Amylose-V Complexes

Application of thermal analysis to studies of starch-lipid interactions has advanced the knowledge of structure and thermal behaviour of amylose-lipid complexes in the past decade (Gough *et al.* 1985; Biliaderis *et al.* 1986; Kugimiya *et al* 1980; Paton 1987; Biliaderis *et al.* 1985; Morrison 1985; Eliasson and Krog 1985; Stute and Koneiczny-Janda 1983). The presence of amylose-lipid complexes in starch systems is revealed by endothermic transitions at temperatures well above the melting endotherms of starch crystallites (95-130^{0}C). Complexation is thermoreversible, as evidenced by an exotherm on the DSC cooling curves

(Biliaderis *et al.* 1985; Eliasson and Krog 1985). This transition displays marked thermal hysteresis (ΔT 15-25^{0}C; cooling rate 3-10 ^{0}C/min), as anticipated for nucleation, controlled aggregation processes. The need for molecular nucleation followed by interchain association, results in a temperature region below the melting temperature where crystallization is very slow. Furthermore, the thermal stability of the complexes increases with increasing chain length of saturated aliphatic chains (Morrison 1985: Raphaelides and Karkalas 1988), while it decreases with increasing chain length of cis- unsaturated chains (Raphaelides and Karkalas 1988). In contrast, complexes of trans- unsaturated fatty acids or monoglycerides exhibit similar transition temperatures to their saturated counterparts (Eliasson and Krog 1985).

As early as 1944, Mikus and co-workers determined the location of the complexing agent, and the structure of the complex using X-ray diffraction techniques. They established that the ligand molecules are located inside the helical coil of the amylose chains and not within the interstices between the helices. In the helix, the glucose residues are oriented such that the hydroxyl groups are directed outwards from the helix (Rundle *et al.* 1944). As a result, the interior of the helix becomes hydrophobic, due to the -CH groups of anhydroglucose residues. Complexation ligands include both polar (such as alcohols and fatty acids) and nonpolar molecules (such as iodine) being held within the helical cavity (Carlson *et al* 1979).

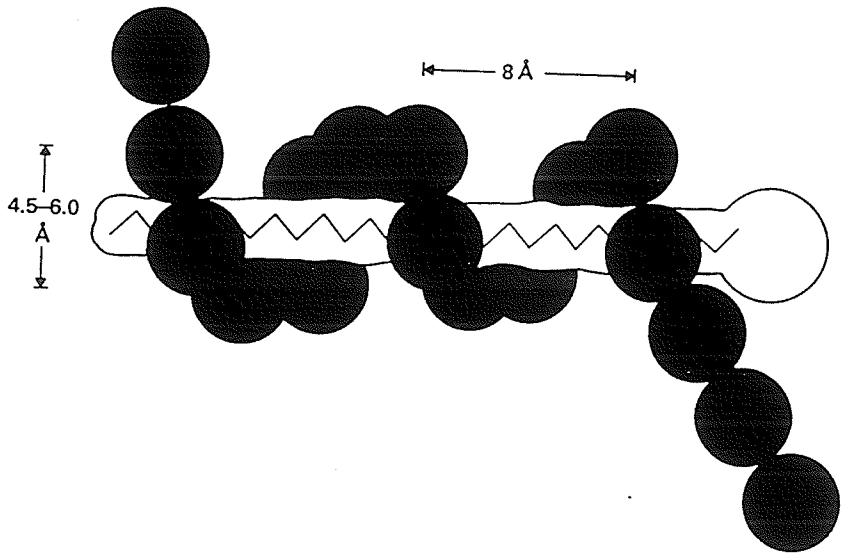
Depending on the size of the complexing ligand the helices can have six (V_6), seven (V_7) and eight (V_8) glucose monomers per turn (Yamashita 1965; French *et al.* 1963). This corresponds to unit cell motifs with

external helical diameters of approximately 13.5, 15.0 and 16.5Å and with internal helical cavity diameters of 4.5, 6.0 and 7.5Å for V_6, V_7 and V_8 amylose complexes respectively. All of these unit cells showed a common fibre repeating distance of 8Å (figure 7). The X-ray diffraction crystallography of these complexes gives (Winter and Sarko 1974; Murphy and French 1975) rise to the so called V pattern of amylose, with reflection peaks at 7.36, 13.1 and 20.1 $2^0\theta$.

Several of the studies on crystal structure of V-amylose complexes also suggested that these complexes form lamellar crystals, having many features in common with lamellar structures of linear synthetic polymers (Manley 1964; Zobel *et al.* 1967; Yamashita *et al.* 1973). Lamellar structures were described for the complex, since the thickness of the crystal platelets were considerably less than the length of the extended amylose helix (Rundle and Edwards 1943). This type of lamellar habit was typical for linear macromolecules crystallized by chain folding at a relatively slow rate. In most lamellae the chain-folded macromolecules were assumed to lie parallel to the growth faces of the crystals (Wunderlich 1973). Furthermore, since the macromolecular chain axes are always at right angles to the lamellar surface, the lamellae thickness is closely related to the chain folded length.

The basic structural units (helix) of the amylose complexes are presumed to be stabilized by a composite of several interactions; i.e. hydrophobic forces between amylose and guest molecules; interchain hydrogen bonding between glucose O-2′——O-3′ molecules and interturn hydrogen bonding between O-2——O-6 and O-3——O-6 molecules (Hinrich *et al.* 1988). Furthermore, water molecules surrounding the structure

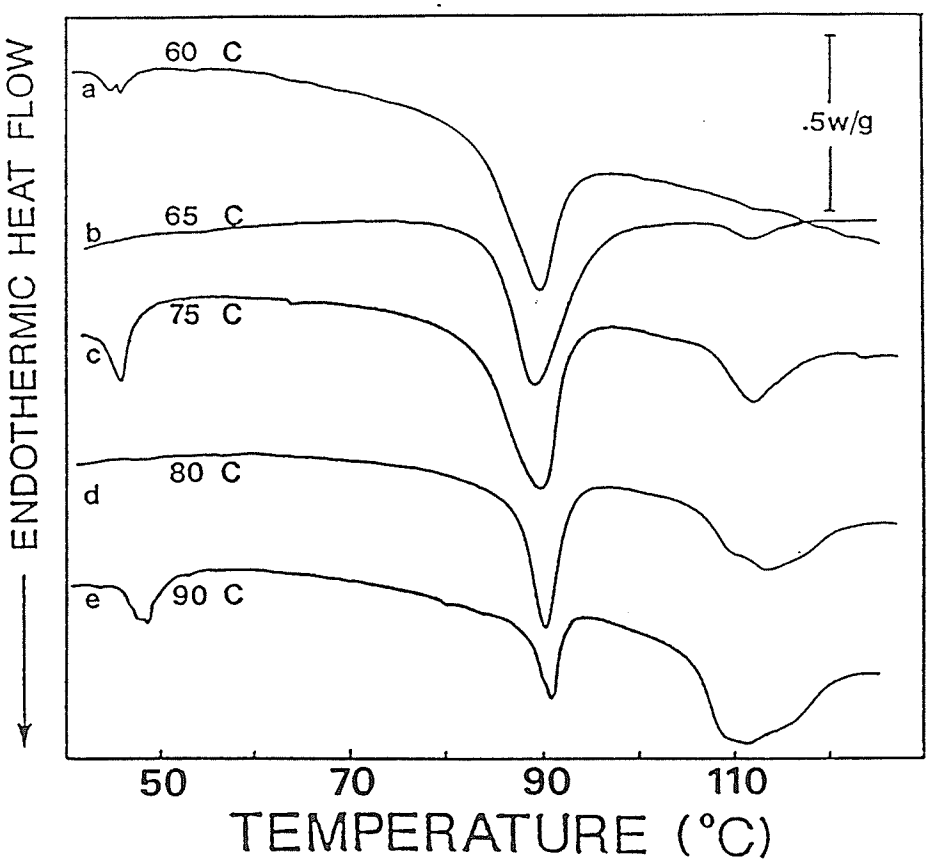
Figure 7 . Representation of the amylose-lipid complex in which a C_{18} saturated fatty acid chain occupies the core of a helix containing six anhydroglucose units (solid circles) per turn (Takeo and Kuge 1969).


8 Å
4.5–6.0
Å

contribute considerably to the stability of the structure. Consequently, changes in the solvent quality can have substantial effect on the conformational stability of the complex.

In addition to X-ray diffraction data, the electron diffraction data (Yamashita 1965) as well as structural studies involving α-amylase digestion and gel permeation chromatography on resistant amylodextrin fragments (Jane and Robyt 1984) have suggested a lamella like organization for V-amylose. However, very little is known about the temperature dependence of crystal size and morphology of the complex. Biliaderis and Galloway (1989), and Galloway *et al.* (1989), have recently studied the role of temperature on crystallization of glycerol monostearate-amylose complex in solution or upon extrusion of cereal flour. Furthermore, they provided some insight into the organization and property/structure relationships of these polycrystalline materials using DSC, X-ray diffraction and gel permeation chromatography. The DSC results (figure 8) showed two distinct transitions over a range of crystallization temperatures. The enthalpy of the second endotherm increased with the increase in crystallization temperature. Water content also appeared to influence the DSC thermal profiles of the complexes. At high moisture situations, a single transition was observed, which implied that zero entropy production melting conditions (i.e. melting without changing the metastability of the system) are approached. However, as the water content was decreased (<70% w/w), the peak temperature was elevated and a second transition at a higher temperature progressively evolved; the DSC thermal curves became characteristic of nonequilibrium melting (Biliaderis *et al.* 1985; Biliaderis *et al.* 1986). These observations show that two

Figure 8 . DSC thermal curves of amylose-monomyristin complexes (20% w/w in H_2O) crystallized at various temperatures (60-90^{0}C) (Biliaderis and Galloway 1989).


ENDOTHERMIC HEAT FLOW
60 C
a
.5w/g
65 C
b
75 C
c
80 C
d
90 C
e
50
70
90
110
TEMPERATURE (°C)

structurally distinct forms (i.e. I and II) of amylose-lipid complexes exist depending on the crystallization temperature. Differences in melting temperature (Tm) between forms I and II have been attributed to difference in structure and/or perfection of their crystallites. The results obtained from X-ray diffraction analysis on the complexes are shown in figure 9. According to the diffraction patterns, only form II figure 9(a) exhibited the three distinct V-diffraction lines (at 7.36, 13.1 and 20.1 $2^0\theta$) clearly suggesting the presence of crystalline regions. On the other hand, form I gave patterns of amorphous like material. As to the size distribution of chain segments, studied by α-amylase etching of disordered regions of the complex and gel filtration chromatography of the resistant fragments, only minor differences in the chain lengths were observed between forms I and II. Based on these findings and other rheological, density and birefringence data of these forms it was suggested that structural differences between forms I and II are mainly entropic.

In this regard, a model was proposed for the organization of the helical domains (figure 10). According to this scheme, at low crystallization temperatures (Tc), formation of complex I is the preferred process. The existence of this form, although metastable in character, is determined mainly by kinetics. In the presence of good complexing agents and at low Tc, the nucleation rate is high, thereby causing rapid "freezing" of helical chain segments with very little crystallographic register (form I) throughout the structure. Therefore, interhelical amorphous chain regions would be under considerable strain and it is likely that they would exhibit an elevated glass transition temperature (Tg), very close to melting temperature (Tm) of the helices. This suggests

Figure 9 . X-ray diffraction diagrams of wet amylose-monoglyceride complexes obtained at various crystallization temperatures: (a) 90°C and (b) 60°C (Biliaderis and Galloway 1989).

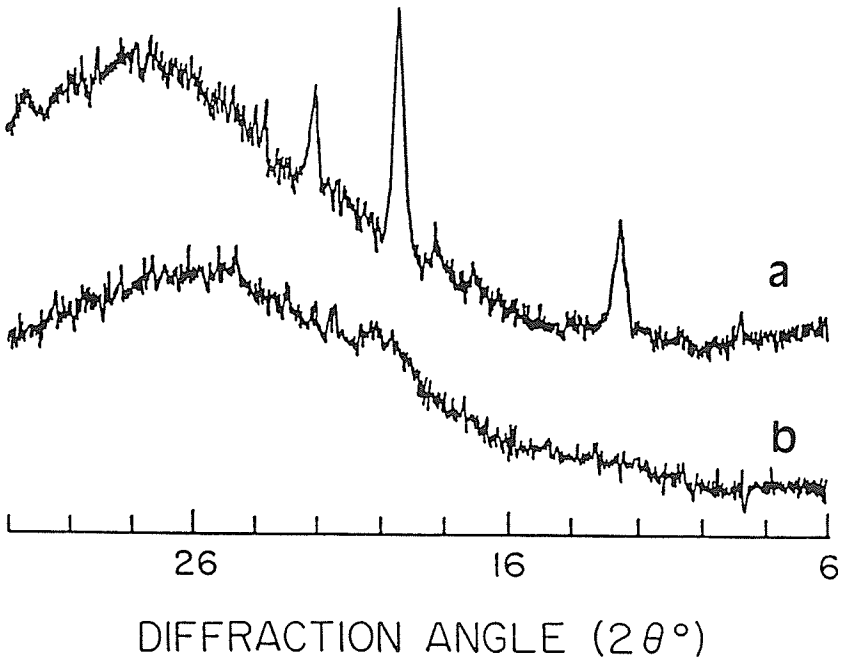
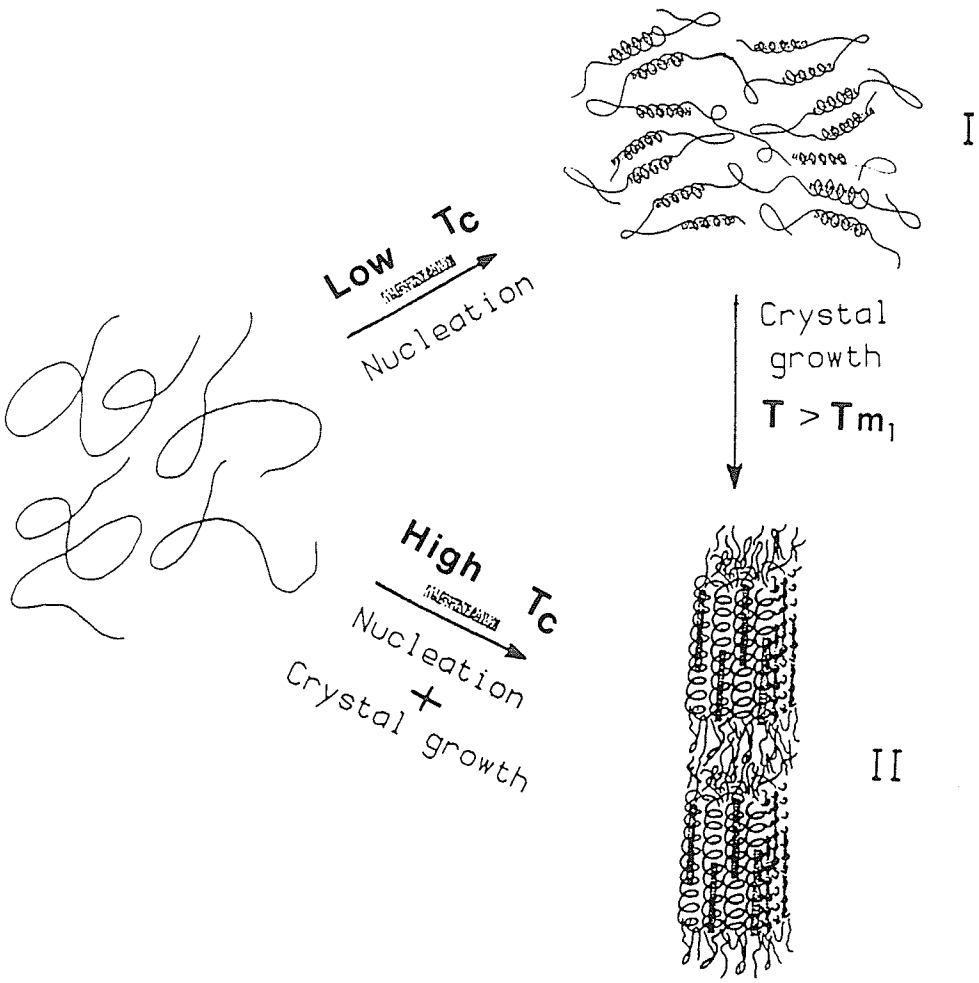

a
b
26
16
6
DIFFRACTION ANGLE (2θ°)

Figure 10. Generalized mechanism for amylose lipid complex formation from dilute solutions which can account for the properties and postulated morphological features of Form I and II (Biliaderis and Galloway 1989).


I
Low Tc
Nucleation
Crystal
growth
T > Tm1
High Tc
Nucleation
+
Crystal growth
II

that high energy barriers exist between the two forms. On the other hand at high Tc, nucleation is a slower process followed by crystal growth to result in form II which has a partially crystalline structure. It should be pointed-out that there is a need to know more about the supermolecular organization and parameters controlling it (i.e. temperature, solvents) since the supermolecular structure state is the functional state of V-amylose complex in starch based products of food.

Solvent Effects on Macromolecules

Biological macromolecules are composed of one or more linear polymer chains each containing monomers which are covalently linked from end to end. There are also non-covalent intra- and interchain interactions operating within the limitations, imposed by monomer sequences, which are responsible for the secondary, tertiary and quaternary molecular conformation. In this regard, the equilibrium conformation adopted by a macromolecule becomes a sensitive function of residue composition and sequence as well as solvent environment it is present (von Hippel and Schleich 1969). Despite considerable work devoted to this subject, there has been little progress in understanding the underlying phenomena to these effects. However, at equilibrium, the macromolecules seem to cluster around the conformation of lowest free energy. For macromolecules, which conformation represents that of lowest free energy as well as how other conformational energy levels are distributed are greatly dependent on solvent environment. Thus, modification of the solvent can dramatically

alter the structure and conformation of macromolecules.

Salt Effects on Stability of Macromolecules

Neutral salts have been defined as strong electrolytes which are significantly soluble in water without changing the pH of the solution (von Hippel and Schleich 1969). Such salts can influence electrostatic interactions in macromolecules by a simple charge shielding basis. In addition to such non-specific effects, neutral salts also exhibit strikingly specific effects on the conformation of macromolecules which seems to have nothing to do with the sign or magnitude of the ionic charge. Hofmeister first arranged various electrolytes on a hierarchical series based on the effectiveness to induce salting-out of euglobulins from solution in an attempt to categorize the influence of specific neutral salts. In subsequent studies with a variety of different electrolytes and other macromolecules (von Hippel and Schleich 1969), the sequence seemed to follow the pioneering work of Hofmeister. In this context, the characteristic ranking of ionic effectiveness in promoting stability of macromolecular conformation has been known as the Hofmeister or lyotropic series.

In addition to bringing about protein precipitation, the position of an ion within the Hofmeister or lyotropic series is related to the ability of an ion to stabilize or destabilize a protein, activate or inactivate enzymes and gelatinize starch granules. For example, increased concentration of salts such as Na_2SO_4 are effective in precipitating

proteins and in stabilizing starch granular integrity. In contrast, high concentrations of salts such as NaSCN or NaI, which increase salting-in or solubilization of proteins, also promote diverse conformational disturbances in other molecules (von Hipple and Wong 1965). However, a complete mechanistic explanation of the effects of neutral salts on macromolecules is difficult due to the complexity of multicomponent interactions.

One approach developed to explain the phenomena for protein systems classifies, neutral salts as either chaotropic or non-chaotropic. Non-chaotropic salts are those which had a stabilizing influence on the protein structure. These salts are assumed to be promoting the ordering of water such that exposure of nonpolar groups becomes less thermodynamically favourable (Hatefi and Hanstein 1969; Hanstein *et al.* 1971). Thus, an increase in the concentration of non-chaotropic salts results in intermolecular hydrophobic associations and precipitation of macromolecules. In contrast, chaotropic salts show little influence on the order of water. Hatefi and Hanstein (1969) defined chaotropic ions as charged groups which transfer, nonpolar groups to water; as a result, in a chaotropic medium the macromolecule destabilizes and exhibits a high solubility due to the transfer of hydrophobic residues into the solvent medium.

Another explanation given to the behaviour of salts was related to the capacity of the salt to raise the surface tension of water. A correlation has been shown to exist between the anionic lyotropic series and the molar surface tension (σ). The molar surface tension is a numerical index reflecting the ability of a salt to alter the surface

tension of water. In a study by Arakawa and Timasheff (1982), the stabilizing effect of certain salts has been attributed to the ion's capacity to increase the water surface tension together with their ability to induce preferential hydration. Preferential hydration, resulting from a specific solvent-salt interaction, makes the immediate area surrounding the macromolecule to become devoid of salts or to contain reduced amounts relative to the bulk solvent (Lee and Timasheff 1981); this is an extension of non-chaotropic concept. According to Arakawa and Timasheff (1982), with respect to stabilizing salts, the concentration required to cause preferential hydration of macromolecules also causes an increase in the solvent cohesive forces. As a result, a high level of energy will be required to convert the molecule into an unfolded structure in such medium; consequently, the folded conformation is favoured.

For proteins, the chaotropic salts do not induce preferential hydration at high concentrations, but instead bind directly to the protein and cause protein-ion interactions (Arakawa and Timasheff 1982). Thus, electrostatic repulsion forces will prevent intermolecular aggregation, and as a result have a salting-in effect, whereby the solubility of the protein is increased.

With respect to denaturants such as urea and guanidinium chloride, there is no generally accepted explanation for their effectiveness. Initial rationalizations focused upon their potential for hydrogen bonding where they were considered to break protein hydrogen bonds. However, further experimental data suggested that they were no more potent in this respect than water (Tanford *et al.* 1962). Furthermore, model compound studies demonstrated that both urea and guanidinium chloride increase the

solubility of non-polar molecules in proportion to their accessible surface area, diminishing the magnitude of hydrophobic effect by up to one third. Although this effect was adequate to produce unfolding of proteins, the observable effects were considerably less than the predicted. As a result, it was assumed that there must be additional factors, probably direct interactions with proteins. It is most likely that denaturants such as urea and guanidinium act indirectly by diminishing the hydrophobic interactions in a uniform manner, and that they directly interact with both the folded and unfolded state of proteins to produce a wide range of effects, depending upon the local geometry of the interacting groups in the protein (Tanford 1973).

Although much of the work on solvent effects has been carried out on protein systems and many theories have been developed for this effects, Erlander (1968) and Mangels and Bailey (1933), observed similar trends for the gelatinization of starch granules. For example, the relative gelatinization power of sodium salts corresponded to the Hofmeister series. In this context, the effects of sodium sulphate, sodium nitrate and also urea correlated with their positions in the Hofmeister series. Furthermore, the mid-point temperature (Tm) of the disorder-order transition increases systematically with the Hofmeister number for the anion through the lyotropic series for the polysaccharide kappa-carrageenan gels (Norton *et al.* 1984).

The observation that the effects of anions on the stability of native structures of many unrelated biopolymers (proteins, nucleic acids, as well as polysaccharides) follows a common sequence suggests that the action of anions is brought about through modification of the water

structure. On this basis, one might similarly suggest that the effectiveness of various small solutes in facilitating starch gelatinization is associated with their capacity to break down the intermolecular order in water.

Effects Of Sugars on Stability of Macromolecules

The effects of different sugars on the structure and functional properties of starch granules have been investigated by many researchers (Osman 1975; Bean *et al.* 1978; Wootton and Bamunuarachchi 1980). In general,various sugars, including sucrose, fructose and glucose raise the gelatinization temperature and delay the increase in the viscosity ("pasting") of starch in starch/sugar/water systems; this effect increases with increasing concentration. Many explanations have been given for this behaviour including competition for water, lowering of water activity and interactions of sugars with the amylose component of the granule (Spies and Hoseney 1982; Lund 1984). However, it has been difficult to rationalize all the results on the basis of a particular theory.

An alternative approach has been taken by Slade and Levine (1984) to explain the elevation of starch gelatinization temperature by sugars. They have suggested that a glass-rubber transition precedes the melting endotherm of starch, thus the melting is controlled by the mobility of the amorphous material surrounding the crystallites. As with other synthetic polymers, melting can proceed only after exceeding the characteristic glass transition temperature (Tg) of the glassy regions of the granules.

According to these workers, a water-sugar mixture is less effective in depressing the Tg of starch, relative to water alone (i.e. sugar-water exerts an antiplasticizing action). In view of the molecular weight dependence of Tg for polymeric materials, they further suggested that antiplasticization is enhanced by increasing the molecular weight of the cosolvent within a homologous series from monomer to dimer to oligomer to polymer. Experimental data on gelatinization of starch confirmed this prediction.

In a subsequent study conducted by Slade and Levine (1987), with a homologous series of sugars, the DSC results have shown that the temperature of gelatinization increases in the following order: water alone< galactose< xylose< fructose< mannose< glucose< maltose< lactose< maltotriose< 10-DE maltodextrin <sucrose. Thus it was concluded that no single parameter can explain completely the mechanism of elevation of Tg by sugars. Instead a combination of these parameters provides useful information to explain why the elevation effect on Tm is greater for sucrose than maltodextrin and glucose than fructose.

Enzyme Digestion of Starch

The digestion of starch by enzymes has been reviewed by several authors including French (1957), Pazur (1965), Manners (1979) and Robyt (1984). According to the mechanism of action, hydrolysis of starch by enzymes involves the addition of water to the α-D-glucosidic bond, and catalysis occurs on the surface of the enzyme. The active site, is divided

into two parts (1) the binding site, made up of a number of subsites and (2) the catalytic site, made up of two groups that represent a proton donor (electrophile) and a proton acceptor (nucleophile) (Pazur 1965). The number of subsites and their arrangement in conjunction with the catalytic groups determine the type of hydrolysis products formed. In the formation of the enzyme substrate complex the α-D-glucosidic bond oxygen is protonated by a hydrogen ion from an amino or imidazole group, of the enzyme and the electron deficient centre at C-1 of the bond attaches an electron from a carboxyl group of the enzyme. The resulting strained structure is cleaved on the C-1 carbon side of the bond, forming a carbonium ion intermediate and a neutral α-D glucosyl fragment. The final step involves the addition of a hydroxyl ion (or water molecule) to the carbonium intermediate (Pazur 1965).

Enzymes Involved in Hydrolysis

In general, α-amylases are endoenzymes attacking α-glucans away from the chain ends at an internal glucosidic bond. They effect the rapid fragmentation of starch molecules by cleaving the α-D-(1-4)-glucosidic linkages and bring about extensive conversion of starch into a variety of reducing sugars and relatively low molecular weight limit dextrins (Robyt and French 1963; 1967; Banks and Greenwood 1975). As a result α-amylase produces a rapid decrease in iodine stain but only a comparatively small increase occurs in the reducing power. The α-amylases are produced by many different bacteria, fungi, animals and some plants. The action patterns

of α-amylases have been widely studied and those for α-amylase from hog pancreas and *Bacillus subtilis*, used in this study, are two such examples.

B. subtilis α-amylase is a dimer protein with molecular weight of 96,000. This α-amylase is a calcium metallo-enzyme with four calcium ions per enzyme molecule (Robyt 1984). Inactivation of the enzyme by removing the calcium is reversible. Enzyme activity is inhibited by heavy metal ions (i.e. Hg) and calcium chelating agents. Optimum pH is between 5.0-6.0. Analysis of the type of starch hydrolysis products produced by this enzyme shows the formation of maltose and maltohexaose. It was postulated by Robyt and French (1963) that the enzyme has a binding surface consisting of 9-D-glucosyl subsites in which the catalytic groups are positioned between the third and fourth subsites from the reducing end subsite. The enzyme functions optimally when all subsites are filled, however, the smallest linear oligosaccharide capable of reaction is maltohexaose.

Hog pancreas α-amylase is a glycoprotein with a molecular weight of 45,000. It contains one calcium ion per enzyme molecule which acts to stabilize the ternary structure which is necessary for its catalytic function. The presence of chloride ion is also required for its catalytic activity (Robyt 1984). Enzyme activity is inhibited by heavy metal ions and calcium chelating agents. Optimum pH is 7.0 and the isoelectric point is 5.5. Analysis of the type of starch hydrolysis products, produced by this enzyme, shows specificity for the formation of maltose and maltotriose. It was postulated by Robyt and French (1967) that the enzyme has a binding surface consisting of 5-D-glucosyl subsites and that the catalytic groups are located between the second and third subsite from the

reducing end subsite. The enzyme functions optimally when all subsites on the binding surface are filled.

Factors Effecting Enzyme Availability of Starch

According to numerous investigations, a wide variation in digestibility of native, gelatinized and chemically modified starches depends on the source of starch and food processing and storage conditions (Dreher *et al.* 1984; Preiss and Levi 1980). However, many digestibility studies produce inconclusive results. These have been attributed to artifacts introduced during sample preparation or improper control of important variables governing the kinetics of heterogeneous reactions, including particle size, porosity, extent of physical or chemical modification and structural homogeneity etc.

It is generally accepted, however, that intact granular (raw) starches are less digestible than their gelatinized counterparts. Furthermore, cereal starches (A-type) are assumed to be more easily digested than root or tuber starches(B-type). Cooking greatly improves the digestibility of poorly digested starches, presumably due to granular disorganization and changes in the crystallinity. The digestibility also depends on the form of food, i.e. particle size and food texture (porous vs. compact). Specifically, products containing whole cereal grains show slow digestion rates *in vitro*, and are also efficient in reducing the glycaemic responses. Reasons given for this observation is the low ratio of the surface area to starch and that the dietary fibre in whole cereal

grains is present in the cell walls forming an intact structural network which encapsulates starch and thereby restricts the accessibility of enzyme to starch. Incompletely gelatinized products, made from whole wheat by various industrial processes (extrusion cooking, popping, steam flaking and drum drying), also exhibited reduced rates of α-amylolysis (Holm *et al.* 1985, 1988) and low glycaemic responses (Holm *et al.* 1985).

Although much work has been done on this aspect the manner by which these factors affect enzyme digestion of starch is relatively unclear. According to many investigators, internal amylose-amylopectin content influences the enzyme susceptibility (Berry 1986). High amylose maize starch was poorly digested in both humans and mice (Wolf *et al.* 1977). Furthermore, enzyme hydrolysis is presumed to have heterogeneous mechanisms where the accessibility and crystallinity are the main limiting factors (Gallant *et al.* 1972).

Studies have also shown that retrogradation of starch in cooked products decreases its susceptibility to hydrolysis (Kayisu and Hood 1970), thus rendering some starch undigestible to α-amylase (Berry 1986; Bjorck *et al.* 1986; 1987). This form which mainly involves the amylose component, is currently referred to as "resistant starch" (RS), and is generated during heat processing. Furthemore, amylose content and yields of RS formed by wet autoclaving/cooling of starches are positively correlated (Berry 1986; Sievert and Pomeranz 1989). The formation of RS in commercially available corn, potato and leguminous purees, intended for infant food, was greater with products containing appreciable amounts of legumes (Siljestrom 1989), probably due to the high amylose content in legume starch than cereals. In contrast to amylose, however, retrograded

amylopectin is completely degraded by α-amylase (Ring *et al.* 1988).

Interaction of starch with other components also play an important role in the digestibility of starch. Specifically, complexation between lipids and amylose which readily takes place during heat processing of starch (Holm *et al.* 1983; Mercier 1980: Schweizer *et al.* 1986) has been invoked as a factor affecting starch digestibility. Recently, a close relationship was observed between the degree of α-amylolysis and the amount of complexed starch in drum-dried and extruded wheat flours. It has also been proposed that the complexes could be regarded as part of the dietary fiber due to low enzymic availability (Larsson and Miezis 1979). Although it has been suggested that such complexes may contribute to the non digestible fraction of polymeric carbohydrate presents in processed foods (Larsson and Miezis 1979), the studies of Holm *et al.* (1983) showed a complete digestion of the V-complexes in the gastrointestinal tract of mice and in the presence of large excess of enzyme. However, the plasma glucose and insulin responses following the ingestion of complexed amylose were significantly lower than the ingestion of free solubilised amylose. Consequently, the complexed amylose was essentially completely absorbed, but at a slower rate compared with free amylose (Holm 1988). The rate and the extent to which α-amylase hydrolyse amylose-monogyceride complexes *in vitro* are also influenced by the type of complexing agent, its chain length and unsaturation of the aliphatic chain (Eliasson and Krog 1985). However, there is a need for better designed experiments, using well-characterized complexes, to establish relationships between α-amylase susceptibility and structure/physical properties of these materials.

MANUSCRIPT I

ON THE SUPERMOLECULAR STRUCTURE AND METASTABILITY OF GLYCEROL MONOSTEARATE-AMYLOSE COMPLEX

INTRODUCTION

Experimental data on the melting of macromolecular crystals are often indicative of irreversible (non-equilibrium) processes. As a result, crystal reorganization or recrystallization can occur before final melting. This behaviour is a direct manifestation of the metastability of partially crystalline states, and, for synthetic linear polymers, it has been attributed to small crystal size, chain-folding, and defects in the crystal structure (Wunderlich 1973; 1980). Such non equilibrium states exhibit much lower melting temperatures than equilibrium crystals, and their metastability can be assessed by calorimetry (Wunderlich 1980).

Differential scanning calorimetry (DSC) measurements of phase transitions of starch (Maurice *et al.* 1985, Biliaderis *et al.* 1986a) and amylose-lipid complexes (Stute and Konieczny-Jande 1983; Biliaderis *et al.* 1985, 1986b) have also shown that the thermal curves do not reflect the initial crystallite distribution or the morphology of the crystallites. Instead, when the amount of water present in the system is insufficient to facilitate a co-operative melting path or a moderate heating rate is employed to allow annealing during heating, or both, melting and reorganization can occur simultaneously, which thus yields composite thermal profiles. Approximation of zero-entropy production melting (i.e. melting with out changing the metastability of the system) is, therefore, essential if one is to deduce the degree of metastability and information regarding the structure of original crystallites from their melting

behaviour. For aqueous starch systems, crystallite melting approaches zero-entropy melting conditions in excess moisture situations and under relatively fast heating rates (Biliaderis *et al.* 1986b). One limit of the method of fast heating, however, is the possibility of excessive superheating (the rate of heating exceeds the rate of response of the system), particularly with crystals of more extended chain character and a large number of tie (strained) chain segments (Wunderlich 1980). The DSC data of Shiotsubo and Takahashi (1984) on potato starch gelatinization indicates that, at heating rates above 0.5^{0}C/min, crystallite melting is limited to kinetic limitations (e.g. water diffusion, thermal lags) since Tm increases with the heating rate. Nevertheless, DSC measurements 5-10^{0}C/min in conjunction with excess moisture (80%) do provide useful qualitative information about the structure and metastability of the initial sample, particularly for amylose-lipid complexes (Biliaderis *et al.* 1985; 1986a,b).

In a recent study of the effects of crystallization temperature on the supermolecular organization of amylose-monoglyceride complexes (Biliaderis and Galloway 1989), crystallized from dilute solution, two thermally distinct forms were identified: form I (low Tm), which shows an amorphous X-ray diffraction pattern and readily undergoes reorganization upon heating, and form II (high Tm), which gives the typical V-type crystallographic pattern. The present paper is concerned with differences in the annealing behaviour, thermal stability, and interconversions between these metastable structural forms of glycerol monostearate-amylose complex in both stabilizing (sodium sulphate and sucrose) and destabilizing (urea, guanidine hydrochloride) aqueous environments.

Furthermore, the effects of CsCl on the structure of the complex, as probed by X-ray and DSC, provided additional supporting evidence to the postulate that forms I and II are two distinct thermodynamic states of different internal energy and entropy. The subject is of practical importance since both forms may be found in processed starch-based food products (bread, parboiled rice, extruded cereals, ect.), depending on their thermomechanical history.

Materials and Methods

Materials

Glycerol monosterate was a product of Sigma Chemical Company (St Louis, MO), and amylose, from potato starch, was obtained from Aldrich Chemical Company (Milwaukee, WI). Guanidine hydrochloride (Gdn. HCL), sodium sulphate (Na_2SO_4), and urea (ACS Chem. reagent grade) were products of Sigma Chemical Company. Cesium chloride (CsCl) was obtained from Pharmacia Ltd (Montreal, CAnada). The molecular properties of the amylose fraction were: $[\eta]$ in 1N KOH 156 ml/g, corresponding to a DP of 1150, iodine affinity of 18.9g I_2/100g, β-amylolysis 83.8±1.1% and λ_{max} of its iodine complex 620 nm. Gel-filtration chromatography of this material on a Sephacryl S-1000 superfine column (2.6 x 90 cm, flow rate 0.4 ml/min) eluted with 40% v/v dimethyl sulfoxide (Me_2SO) in distilled water gave a broad symmetrical chain distribution.

Preparation of the complex

The condition for the preparation of amylose-monostearin complexes were essentially those reported by Biliaderis *et al.* (1985). Complex formation was carried out for 24 hr in aqueous solutions (0.25% w/v

amylose; weight ratio of amylose to added ligand 5:1) maintained at a constant temperature (60^{0}C form I and 90^{0}C for form II). The insoluble complexes were recovered by centrifugation (8,000 g) and washed repeatedly with chloroform to remove the free ligand (as assessed by DSC analysis). Samples used for X-ray diffraction and acid-etching experiments were kept in the hydrated state, whereas those intended for DSC analysis were freeze-dried.

Acid hydrolysis

Acid hydrolysis of the complexes in a heterogeneous reaction was carried out at 40^{0}C by suspending 1.5g of solids in 300 ml 1.2 N HCl under continuous gentle agitation. At specific time intervals, the reaction mixtures were neutralized and centrifuged, and the supernatants were assayed for total carbohydrates using the orcinol-sulphuric acid method (0.1% w/v orcinol in 70% v/v H_2SO_4; Miller *et al.* 1960). The extent of hydrolysis was determined by expressing the solubilized carbohydrates as a percentage of the initial complex. After solubilization with Me_2SO, gel permeation of the amylodextrin residues was carried out on a Sephacryl S-200 superfine column (0.9 x 131 cm, flow rate 19.0 ml/hr) eluted with degassed distilled water (35^{0}C). Carbohydrates in the eluate (orcinol-sulphuric acid; absorbance at 450 nm) were continuously monitored with an autoanalyzer.

X-ray diffractometry

X-ray diffraction analysis was performed on hydrated or lyophilized residues deposited on aluminum holders by using a Philips PW 1710 powder diffractometer equipped with a graphite-crystal monochromator. The operating conditions were; copper K_a radiation, voltage 40 kV, recorder time constant 0.5 s, sampling interval time 0.4 s, recorder speed 10 mm/ $2\theta^0$, and scan speed 0.1 $2\theta^0$/s.

Differential scanning calorimetry

The DSC studies were carried out by a Du Pont 9900 Thermal Analyzer equipped with a Du Pont 910 cell base and a pressure DSC cell. The system was calibrated with indium (Biliaderis *et al.* 1985). A pressure of 1500 kPa with N_2 was used to eliminate pan failure at temperatures above 120^{0}C. All samples were prepared in Du Pont hermetic pans by adding the required amounts of solution to a pre-weighed freeze-dried sample of the complex. In this respect, it is of interest to note that freeze-drying of the hydrated complex did not alter the thermal properties (Tm, ΔH) of both forms I and II. All DSC measurements were carried out at 20% solids and 10^{0}C/min to approximate zero-entropy production melting. The lyophilized samples were kept for at least 2 h to equilibrate with the solvent, before DSC analysis, in order to minimize time-dependent changes in the conformation of the complex at room temperature, particularly for solutions of high concentration of destabilizing agents. For isothermal

annealing studies, the samples were heated in the calorimeter for a specified time-temperature regime, cooled and weighed to ensure no losses of solvent during heating.

RESULTS AND DISCUSSION

Annealing effects and structural considerations

Crystallization of the complex at 60°C and 90°C gave pure preparations of structural forms I (Tm 99.4±0.3°C) and II (Tm 116.6±0.2°C), respectively as shown in figure 11. Although, in a number of calorimetric studies of amylose-lipid complexes, multiple melting transitions have been reported, the underlying causes of this behaviour remained unknown (Wirakartakusumah 1981; Bulpin *et al.* 1982; Donovan *et al.* 1983; Stute and Konieczyn-Janda 1983; Kowblansky 1985; Eliasson 1988; Raphaelides and Karkalas 1988). As a result, no attempt was made to provide a possible morphological model for the description of solid state organization of these materials that could account for such thermal responses. On the basis of a number of DSC, X-ray and structural analysis data, it was proposed that multiple melting transitions of complexes represent metastable states of varying degree of organization of the helical chain motifs in the ordered domains. The thermodynamic and kinetic arguments for their existence were given in detail elsewhere (Biliaderis and Galloway 1989) and remained valid for the discussion of this paper. A striking feature of form I was the consistency of its melting temperature, which suggested that it is well defined state.

In the hydrated state, form I lacks the characteristic diffraction pattern of V-complex (figure 12(a)), as compared with form II, which shows the three major reflection peaks of V-crystals at 7.36, 13.1 and 20.1 $2\theta^0$

Figure 11. Effect of annealing temprtatures (2 hr at the specified temperature) on the structure of forms I(left) and II(right). Top curves are of control (non-treated) samples. Heating rates of DSC experiments 10^{0}C/min, solids content 20% w/w. Mass of complex from top to bottom (mg): (left) 2.05 (control), 2.07, 2.01, 1.92, 1.97, 1.93 and 1.97; (right) 2.09 (control), 1.32, 1.42, 1.28, and 1.46.

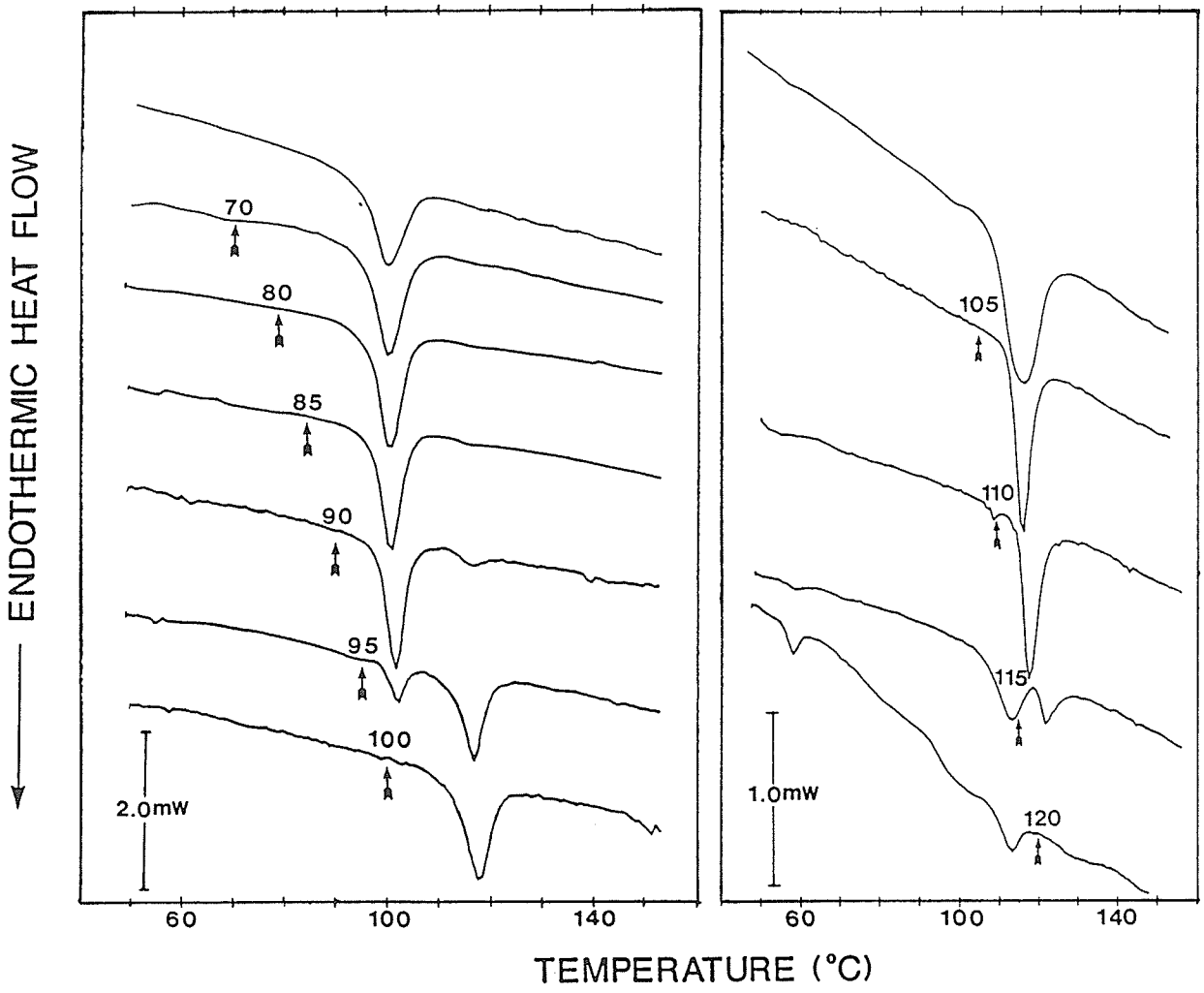
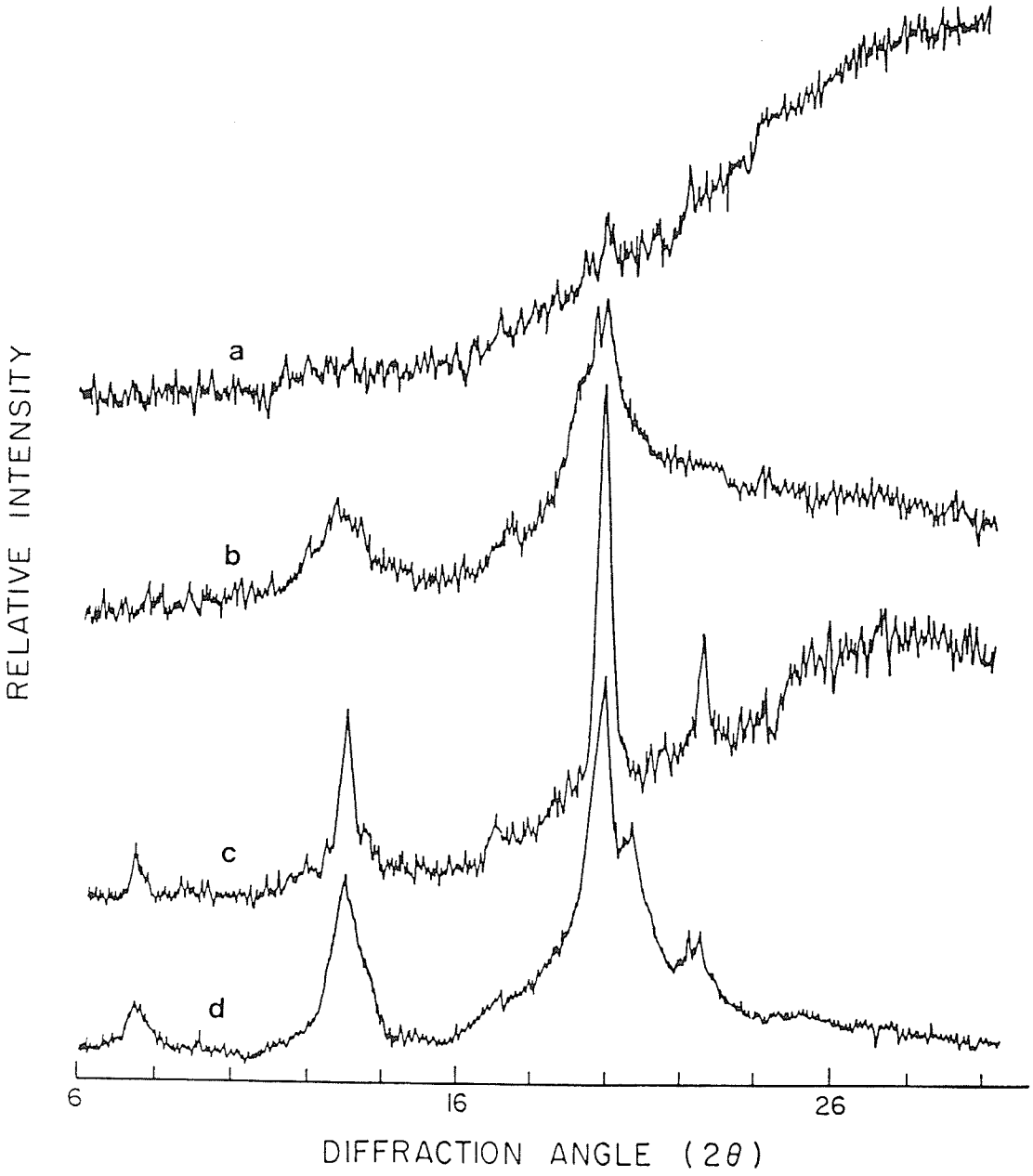

ENDOTHERMIC HEAT FLOW
70
80
85
90
95
100
105
110
115
120
2.0mW
1.0mW
60
100
140
60
100
140
TEMPERATURE (°C)

Figure 12. X-ray diffraction diagrams: (a) form I wet; (b) form I dry; (c) form II wet; (d) form II dry.


RELATIVE INTENSITY
a
b
c
d
6
16
26
DIFFRACTION ANGLE (2θ)

(figure 12(c)). The X-ray diffraction of the dry powder of form I gave some of the characteristic spacings of V-form (figure 12(b)), although diffusely and not as distinct as those of form II (figure 12(d)). It, appears therefore that there is a modification in the structure of form I during lyophilization in that chain aggregates of much higher order are formed, which gives rise to a two line V-pattern. These results are consistent with the view that form I has helices of very little crystallographic register, whereas the structural domains of form II consist of well-developed crystallites. The Tm of form I remains constant regardless of the crystallization conditions (temperature, polymer concentration etc.). However, it is dependent on the chain length of the ligand (Kowblansky 1985; Biliaderis *et al.* 1985; Raphaelides and Karkalas 1988) and the degree of unsaturation of the aliphatic chain (Stute and Konieczny-Janda 1983; Eliasson and Krog 1985). It is also worth commenting here that, despite the difference in X-ray diffraction patterns between the hydrated and lyophilized rehydrated complex of form I (figure 12(a), (b)), the DSC thermal parameters were not altered as a result of freeze-drying, i.e. identical Tm and ΔH values were obtained for the hydrated and dried-rehydrated samples of form I.

The results of the annealing experiments on the structural modification of the complex are shown in figure 11. The effects of annealing temperature on form I is negligible up to about 90^0C, which is the onset temperature for the melting transition of the initial sample. Annealing at higher temperatures resulted in the development of the second endotherm, whose Tm coincided with that of form II. The fact that form II appears only when the metastable form I is annealed at elevated

temperatures implies that high energy barriers exist between the two forms. Hence, unless the structure of form I is partially melted, it would remain practically unchanged at low temperatures, It is likely that the inter-helical chain segments of form I are under considerable strain and thus exhibit an elevated Tg (Biliaderis *et al.* 1986, Slade and Levine 1987), very close to the Tm of the helices. This situation leads to a kinetically stable system. However, once some helical chains of form I melt, the structure relaxes, and crystallization can proceed rapidly around the remaining helices that can act as nuclei. Since conversion of form I→II upon annealing dose not yield another intermediate between the two forms, form I must represent a distinct thermodynamic state.

An increase in the melting temperature of form II along with a decrease in the half-height width of the melting transition was observed after isothermal annealing (figure 11, right). This suggests the development of larger and more perfect crystallites of narrower size distribution, typical of annealing effects on the macromolecular non-equilibrium crystals (Wunderlich, 1980). Assuming a chain-folded (lamellar) macroconformation, as suggested by electron- and low-angle X-ray diffraction work on single crystals and polycrystalline aggregates of V-amylose (Manley 1964, Yamashita 1965, Zobel *et al.* 1967) as well as structural studies by using α-amylase and gel permeation chromatography (Jane and Robyt 1984, Biliaderis and Galloway 1989), crystallite perfection or thickening or both would proceed via increased motion of chain defects and ingestion of tie-chain segments (Buckley and Kovacs 1984). Annealing at temperatures above the Tm of the initial II-crystals yielded multiple melting peaks. Under these conditions, the rate of

nucleation decreases rapidly, and thus crystallization in the metastable melt is restricted; the sample appears to crystallize during subsequent cooling.

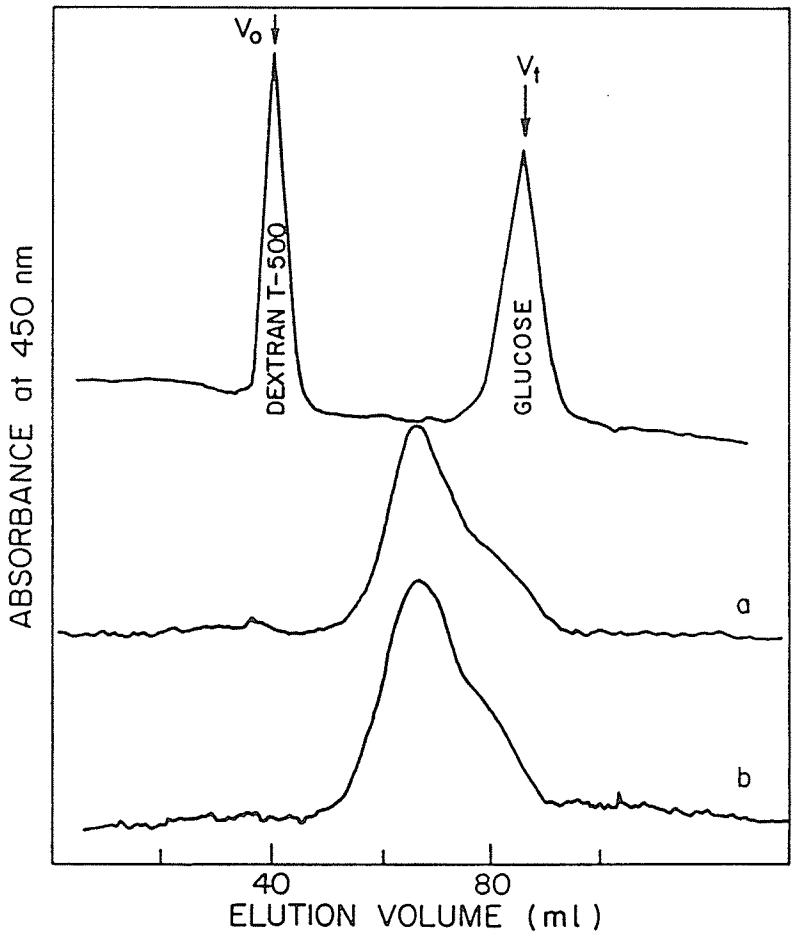
The possibility that differences in the observed Tm between forms I and II might arise from differences in the size of the crystallites, as predicted by the Thomson-Gibbs equation for chain-folded crystals (Wunderlich 1980), was further examined. The chain distribution of the ordered domains in two forms was determined by gel filtration following acid-etching of the complex. Assuming that acid degrades preferentially the amorphous regions (chain folds, loops, and tie molecules), the resistant amylodextrin fragments would represent the chain segments participating in the crystallites. The thermal and chemical properties of the residues at various hydrolysis-time intervals are given in Table 1, and the chromatographic profiles of the six-day treated samples are shown in figure 13. The chain distributions of the resistant fragments of form I (figure 13(a)) and form II (figure 13(b)) were similar despite the different solubilization levels reached at this digestion period. These results are in agreement with previous findings on the characterization of glycerol monosterate and glycerol monopalmitate-amylose complexes by α-amylase etching/gel chromatography (Biliaderis and Galloway 1989). Furthermore, they support the hypothesis that structural differences between forms I and II mainly lie in the degree of organization of the helical chain segments and not in their length (Biliaderis and Galloway 1989).

TABLE 1. Thermal and Chemical Properties of Amylodextrin Residues after Acid-Etching of Glycerol Monostearate-Amylose Complex (form I and II) in a Heterogeneous Reaction Mixture (1.5 g solids/ 300ml 1.2N HCl; 40^0C).

Reaction time (days)	Solubilization (%)		λ_{max} (iodine complex)		ΔH (J/g)	
	I	II	I	II	I	II
1	13.2	15.1	602	606	24.3±1.6[a]	26.8±1.9
2	29.3	27.6	594	599	25.8±0.6	27.5±2.1
6	68.1	42.4	585	577	19.8±1.1	17.9±1.1
12	75.7	54.6	579	580	14.3±3.1	13.2±2.7

[a] n: 3.

Figure 13. Chromatographic profiles of resistant amylodextrin fragments after acid hydrolysis (6 days) of the complex (1.2 N HCl) on a Sephacryl S-200 column (131 x 0.9 cm) eluted with water (flow rate 19.0 ml/hr, 35^{0}C): (a) form I; (b) form II.


V_o
V_t
DEXTRAN T-500
GLUCOSE
a
b
ABSORBANCE at 450 nm
40
80
ELUTION VOLUME (ml)

Solvent effects on conformational stability of the complex

The role played by solvent and small solute-solvent interactions in aqueous solutions of macromolecules is in some aspects far from clear. In general, solutes affect macromolecular stability and conformational interconversion equilibria by direct interactions with the macromolecule and/or by direct action through effects on the structure of water (von Hippel and Schleich 1969). Since the pioneering work of Hofmeister, it has been recognized that neutral salts, for example, drastically alter the solubility and conformation stability of macromolecules; their relative effectiveness follows the lyotropic or Hofmeister ion series (von Hipple and Schleich 1969). Selective interactions between polymer and ion may, however, distort the normal lyotropic order (Rinaudo *et al.* 1979). Furthermore, for polymeric materials having a high structural order, owing to aggregation of chains, salt effects must be interpreted by considering both dissociation of the supermolecular structure and conformational disordering of individual chains. As a result, the ranking may not necessarily parallel the Hofmeister series if the ions act differently at the various levels of ultrastructure, as was shown for reconstituted collagen in the solid state (Chang and Chien 1973; Chien 1975). Incorporation of polyhydroxy compounds (e.g. sugars) is also known to stabilize macromolecules in the solution (Gerlsma 1968; Lee and Timasheff 1981) or in the solid state (e.g. starch; Lund 1984) against the disruptive effect of temperature and pH. In contrast, urea and guanidinium salts are universal denaturants of proteins and other 'native' structures of macromolecules, including starch (Tanford *et al.* 1962; von Hippel and

Schleich 1969).

The thermal responses of forms I and II with increasing concentration of sucrose and Na_2SO_4, used as stabilizing compounds, are shown in figures 14 and 15, respectively. Evidence for structure stabilization of the complex was reflected by increases in Tm for both forms. Furthermore, at high solute concentrations (> 1.17M sucrose, figure 14; >0.7M Na_2SO_4, figure 15), the metastable form I undergoes reorganization to form II during thermal analysis. The exothermic effects between the two endotherms are indicative of recrystallization of the complex into a state of lower free energy (a thermodynamically more stable form). Thus, the thermal curves represent composite effects of secondary processes superimposed on the primary phenomena. It was, in fact, shown that by increasing the heating rate (from 3 to 50^0C/min), the enthalpy of the first transition increased (2.0M sucrose, 2.0M Na_2SO_4), whereas that of the recrystallized material was reduced (data not shown).

The observed stabilizing role of Na_2SO_4 (figure 15) on both structural forms of the complex is in accord with its ranking in the lyotropic series. Sulphate salts [$(NH_4)_2SO_4$, Na_2SO_4] are known to stabilize the native conformation of proteins and nucleic acids (von Hippel and Schleich 1969). Other relevant observations of the influence of anions on swelling and gelatinization properties of starch (Mangels and Bailey 1933; Evans and Haisman 1982) and starch retrogradation (Morsi and Sterling 1963) were also consistent with the lyotropic anion series, $SO_4^{2-} < F^- < Cl^- < Br^- < I^- < SCN^-$; sulphate salts exercise the strongest effect in increasing the gelatinization temperature, retarding swelling, and promoting retrogradation.

Figure 14. DSC thermal curves of structural forms I (left) and II (right) of the complex (20% w/w) in sucrose solutions of varying molar concentrations. Mass of complex from to bottom (mg): (left) 2.46 (control), 2.05, 2.29, 2.02, 2.33, and 2.35; (right) 2.09 (control), 1.72, 1.84, 2.30, and 1.87. Heating rate 10^{0}C/min.

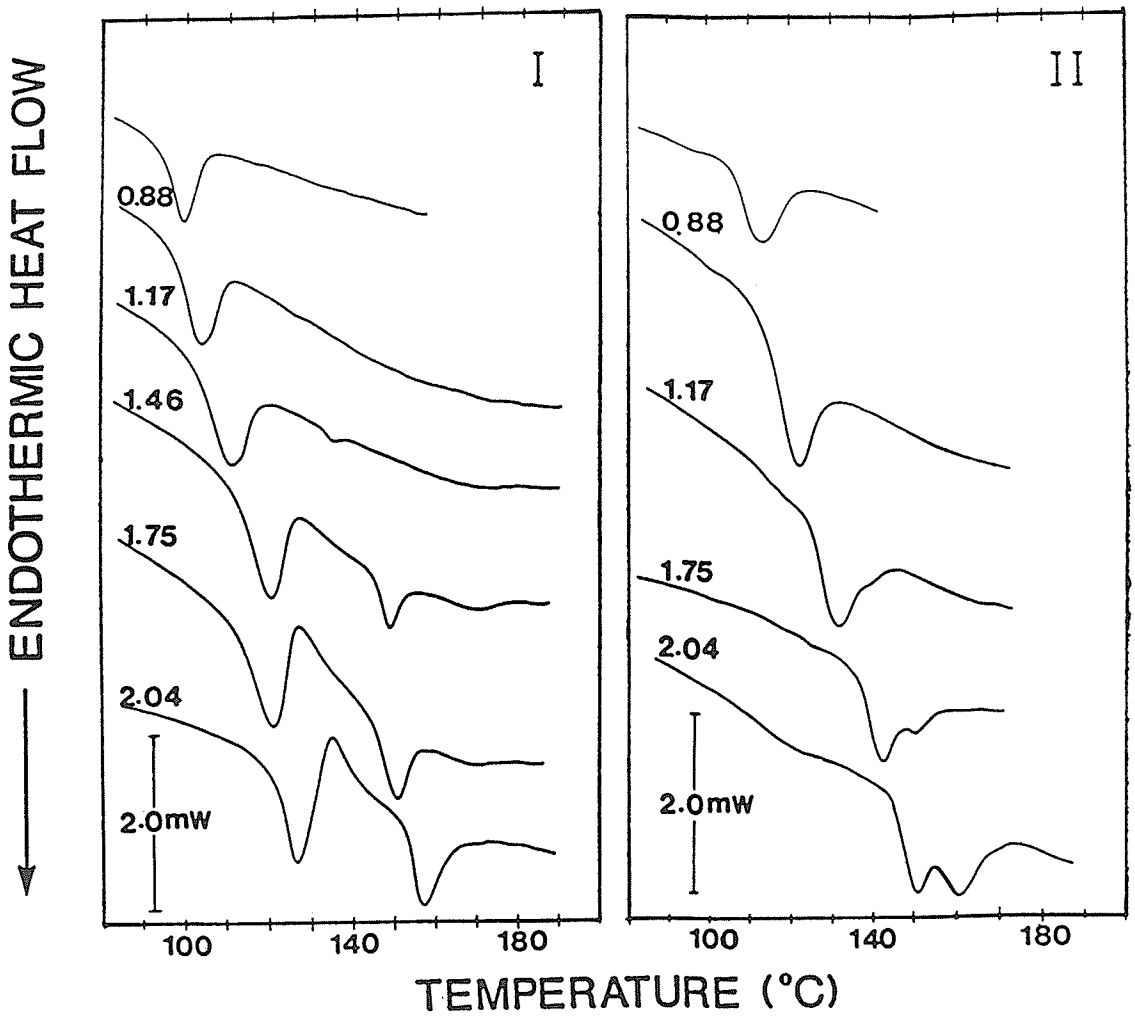
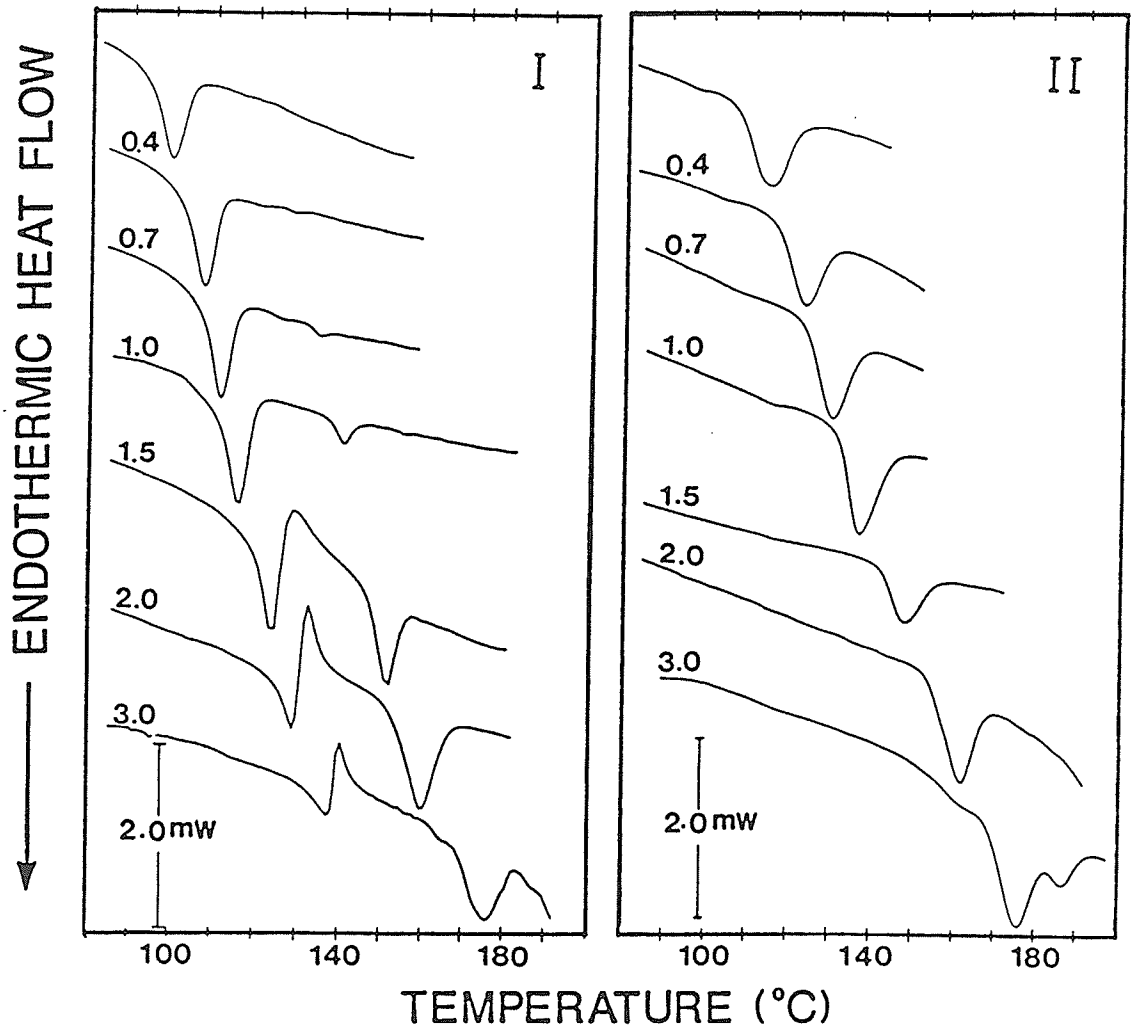

I
II
0.88
1.17
1.46
1.75
2.04
2.0mW
100
140
180
ENDOTHERMIC HEAT FLOW
TEMPERATURE (°C)

Figure 15. DSC thermal curves of structural forms I (left) and II (right) of the complex (20% w/w) in Na_2SO_4 solutions of varying molar concentrations. Mass of complex from top to bottom (mg): (left) 2.05 (control), 2.31, 3.10, 2.39, 2.04, 2.01, and 1.91; (right) 2.09 (control), 1.93, 1.96, 1.97, 1.98, 1.55, and 2.00. Hraeting rate 10^0C/min.


ENDOTHERMIC HEAT FLOW
I
II
0.4
0.7
1.0
1.5
2.0
3.0
2.0mw
2.0mw
100
140
180
100
140
180
TEMPERATURE (°C)

The structure-stabilizing action of sucrose (figure 14) agrees with earlier findings of the effect of sugars on starch gelatinization. Sugars, as well as other polyhydroxy compounds, increase the gelatinization temperature of starch (Bean and Yamazaki 1978a,b; Wootton and Bamunuarachchi 1980; Spies and Hoseney 1982; Evans and Haisman 1982). There has been several interpretations of this behaviour, including competition between starch and sugars for water, inhibition of granule hydration, and specific sugar starch interactions (Evans and Haisman 1982; Spies and Hoseney 1982; Lund 1984). Attempts have even been made to explain and predict the thermal responses of starch in sugar solutions on purely thermodynamic grounds by using an extension of Flory equation to a three-component system (Lelievre 1976; Blanshard 1979; Evans and Haisman 1982). Although this approach provides a convenient framework to analyze melting data, its development is based on equilibrium melting processes and making simplifying assumptions regarding the interaction parameters, X_{ij}, between solvent, solute, and polymer. However, evidence suggesting that melting of starch crystallites is a non-equilibrium process (Slade and Levine 1984; 1987; Maurice *et al.* 1985; Biliaderis *et al.* 1986a; Blanshard 1987) has raised doubts on the use of such a thermodynamic treatment. The observed multiple-melting transitions for glycerol monosterate-amylose complex (figure 14 and 15) at high Na_2SO_4 or sucrose concentration also imply that melting of this material is far from equilibrium or even zero-entropy production melting conditions. Another approach to explain the elevation of the gelatinization temperature by sugars was recently suggested by Slade and Levine (1987). They have considered sugar/water solutions as plasticizing cosolvents that exert less plasticizing effect

on the amorphous regions of starch than water alone. As such, they are less effective in depressing the glass-transition temperature of starch (Tg) than water. This, in turn, leads to an elevated Tm for the crystallites; crystallite melting can commence only after exceeding the characteristic Tg of the surrounding glassy matrix (Slade and Levine 1984; 1987; Maurice *et al.* 1985; Biliaderis *et al.* 1986a).

In contrast to results obtained with Na_2SO_4 and sucrose, the DSC thermal curves of forms I and II in the presence of urea (figure 16) and Gdn. HCl (figure 17) exhibited destabilization of the complex structure. The Tm progressively decreased, and the transitions became broader with increasing concentration of the denaturant. These effects were more pronounced at levels above 0.5M for both agents. At concentrations above 6.0M, a small endothermic transition at 55-58^{0}C evolved, which corresponds to the melting of liberated monoglyceride, presumably owing to the complete disruption of the helices. Urea is also known to reduce the gelatinization temperature of starch and stabilize amylose in solution (Suggett 1974); this behaviour has been suggested to arise from effects on the structure of water. With respect to the order→disorder transition of the structural domains of forms I and II, there were no clear indication that a multi-step pathway is involved under the experimental conditions (heating rate and denaturant concentration) employed, although the melting endotherms did become less cooperative at high concentrations of urea or Gdn.HCl. It is likely that both dissociation of the supermolecular structure and helix-coil transitions occur simultaneously. In addition to Tm, there was also a progressive decrease in the melting enthalpy of the complex with increasing concentration of denaturant

Figure 16. DSC thermal curves of structural forms I (left) and II (right) of the complex (20% w/w) in urea solutions of varying molar concentrations. Mass of complex from top to bottom (mg): (left) 2.05 (control), 2.16, 2.04, 2.45, 2.42, 2.18, and 2.59; (right) 2.09, (control) 2.24, 2.11, 1.93, 2.06, 2.32, and 2.29. Heating rate 10^{0}C/min.

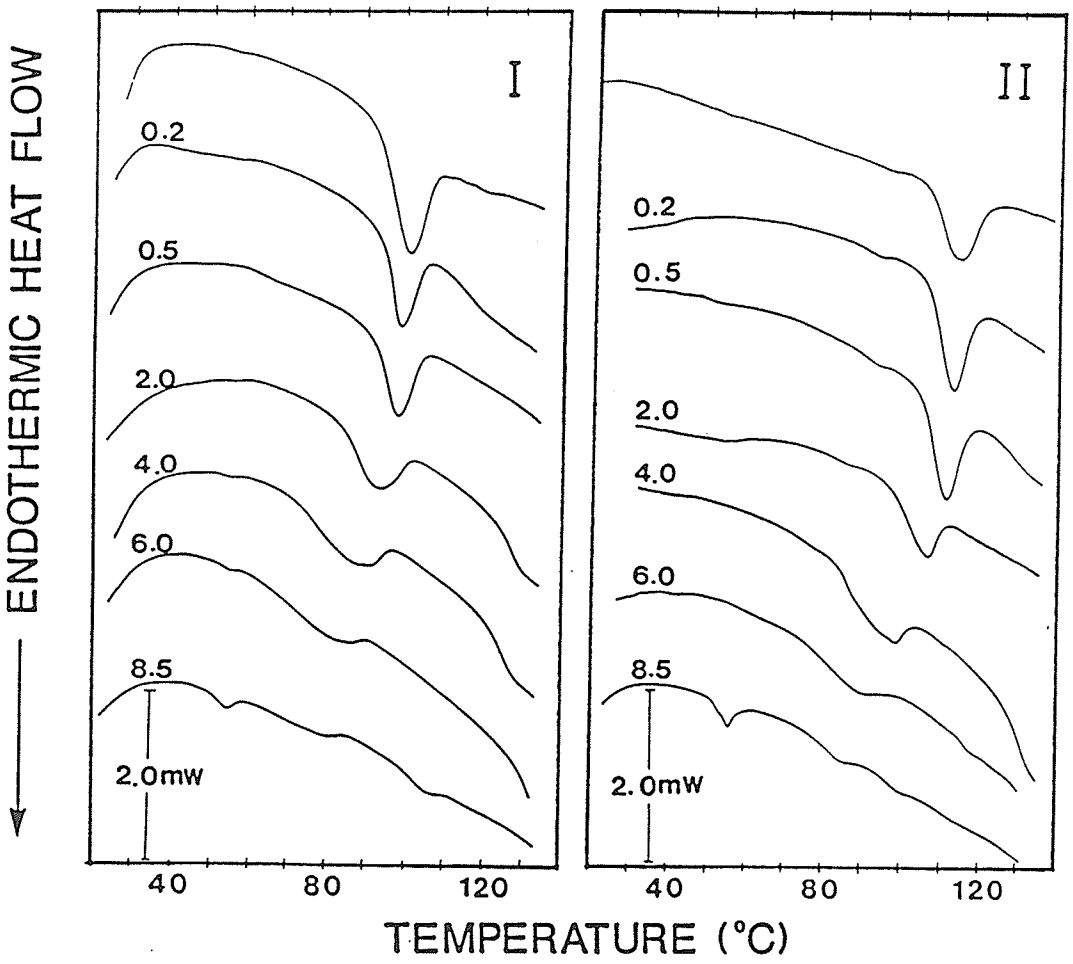
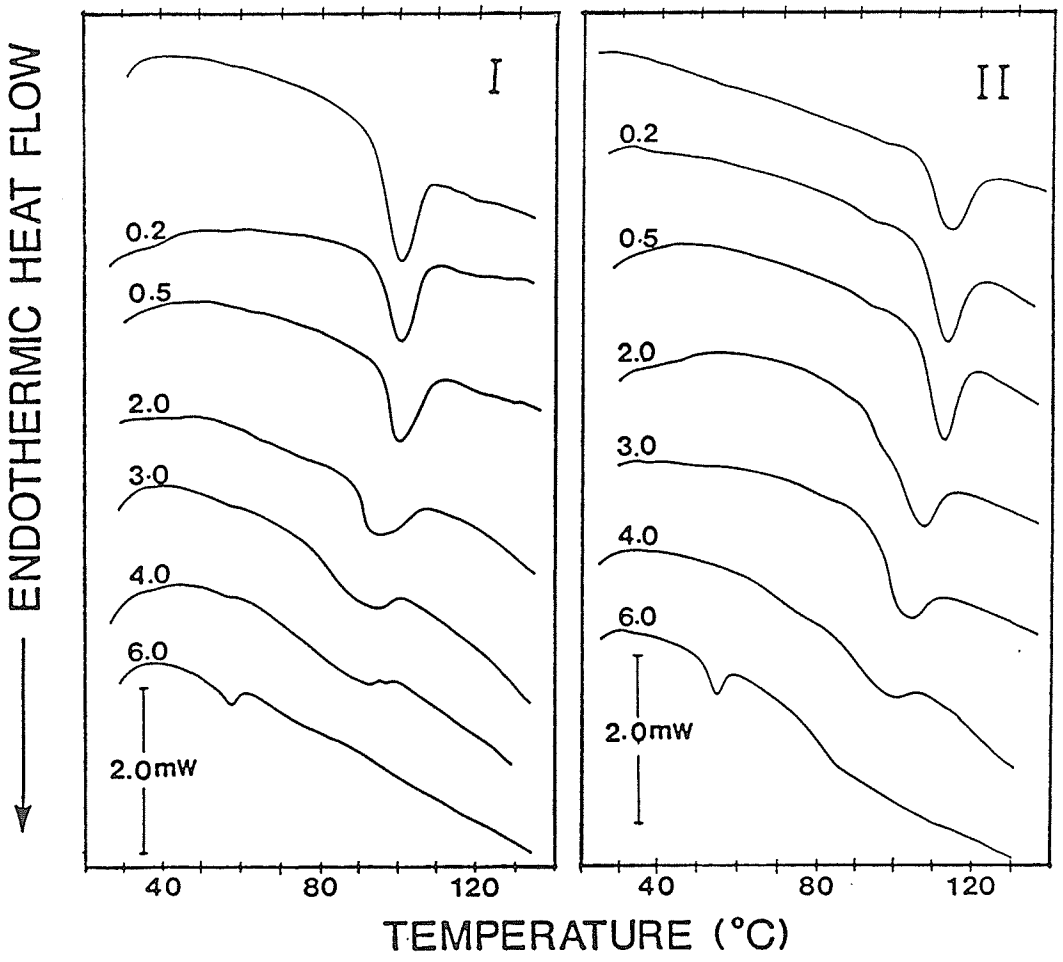

ENDOTHERMIC HEAT FLOW
I
II
0.2
0.5
2.0
4.0
6.0
8.5
2.0mW
2.0mW
40
80
120
40
80
120
TEMPERATURE (°C)

Figure 17. DSC thermal curves of structural forms I (left) and II (right) of the complex (20% w/w) in guanidine hydrochloride solutions of varing molar concentrations. Mass of complex from top to bottom (mg): (left) 2.05 (control), 2.31, 2.27, 1.99, 2.21, 2.31, and 2.21; (right) 2.09 (control), 2.05, 1.96, 1.93, 2.10, 1.96, and 2.45. Heating rate 10^{0}C/min.


ENDOTHERMIC HEAT FLOW
I
II
0.2
0.5
2.0
3.0
4.0
6.0
2.0mW
40
80
120
TEMPERATURE (°C)

(figure 18). Changes in ΔH appeared more rapid in the case of Gdn.HCl than urea, particularly for form I. Interestingly, at low concentrations of Gdn.HCl (3.0 M), the destabilizing potential of guanidinium ion is different for form II, i.e. the transition enthalpy values of form II remained constant, whereas ΔH_1 decreased continuously with increasing molar concentration. Thus Gdn.HCl is relatively ineffective toward the structure of form II. These findings reinforce the idea that there are differences in the supermolecular organization between the various forms of the complex. Urea effects, on the other hand, did not seem to distinguish between forms I and II.

To provide further insight into the structure of the complex, we have examined its thermal behaviour in aqueous solutions of neutral salts by DSC. In these studies, in addition to forms I and II, we used an annealed sample of form II prepared by isothermal annealing (30% w/w complex in water, 120^0C/2hr); to distinguish between these samples, the solution crystallized form II is designated as IIa and the annealed sample as IIb. The X-ray diffraction diagrams of forms I, IIa and IIb along with the corresponding DSC thermal curves are shown in figure 19. The well-defined long-spacing for forms IIa and IIb suggest that these materials have a partially crystalline structure. One can also notice that the intensity of the lines of V-amylose are enhanced significantly with annealing (compare figure 19(b) and (c)). The relative intensities of the diffraction lines at 20.1 $2\theta^0$, taken as an index of long-range order, were: 1.0(I), 8.6(IIa), 15.2(IIb). Despite the marked differences in their supermolecular structure, as evidenced by DSC and X-ray analysis (figure 19), the transition enthalpies of all three forms were of a similar order

Figure 18. Transition enthalpies of structural forms I(a) and II(b) of the complex with molar concentration of guanidine hydrochloride (top) or urea (bottom).

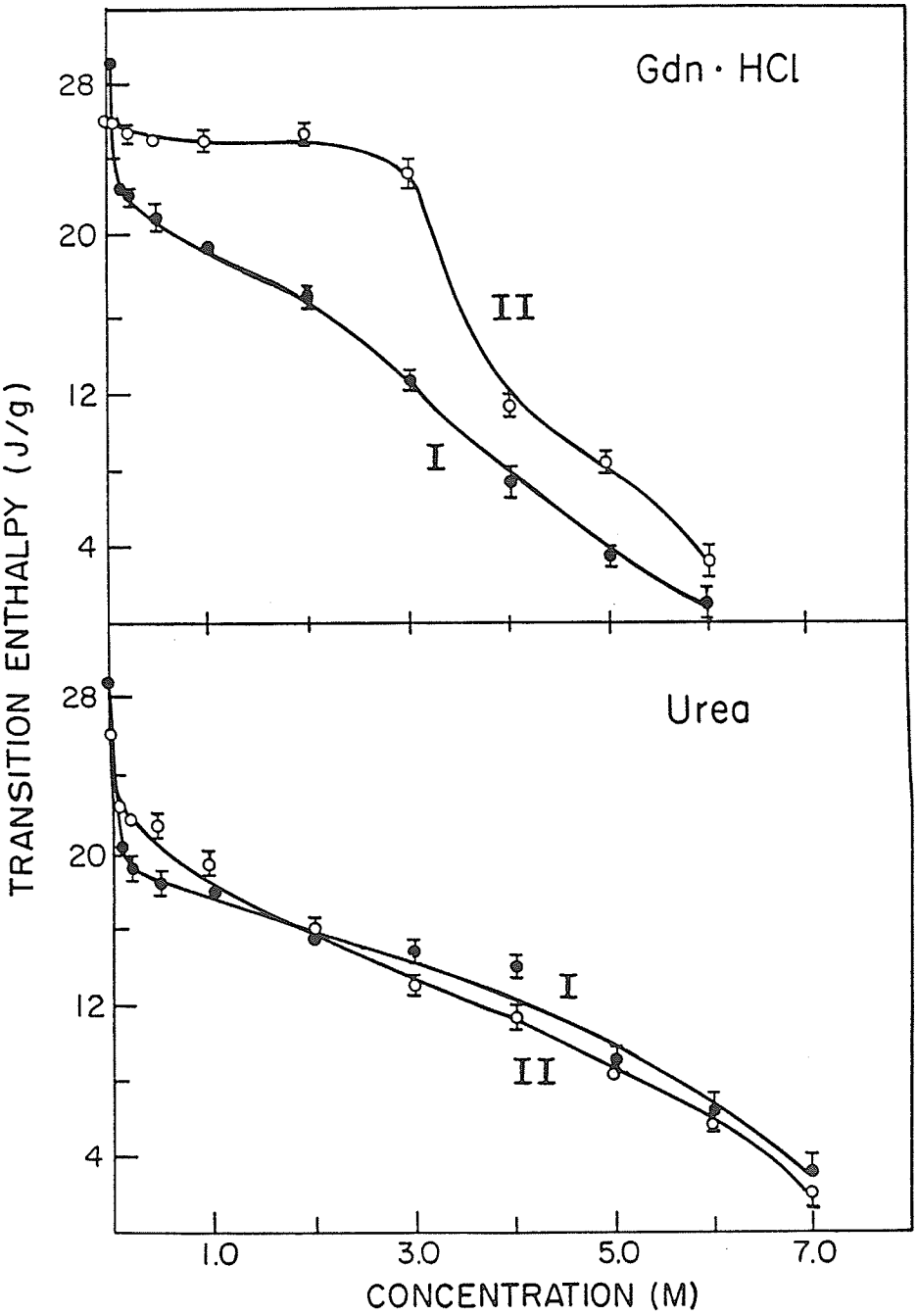
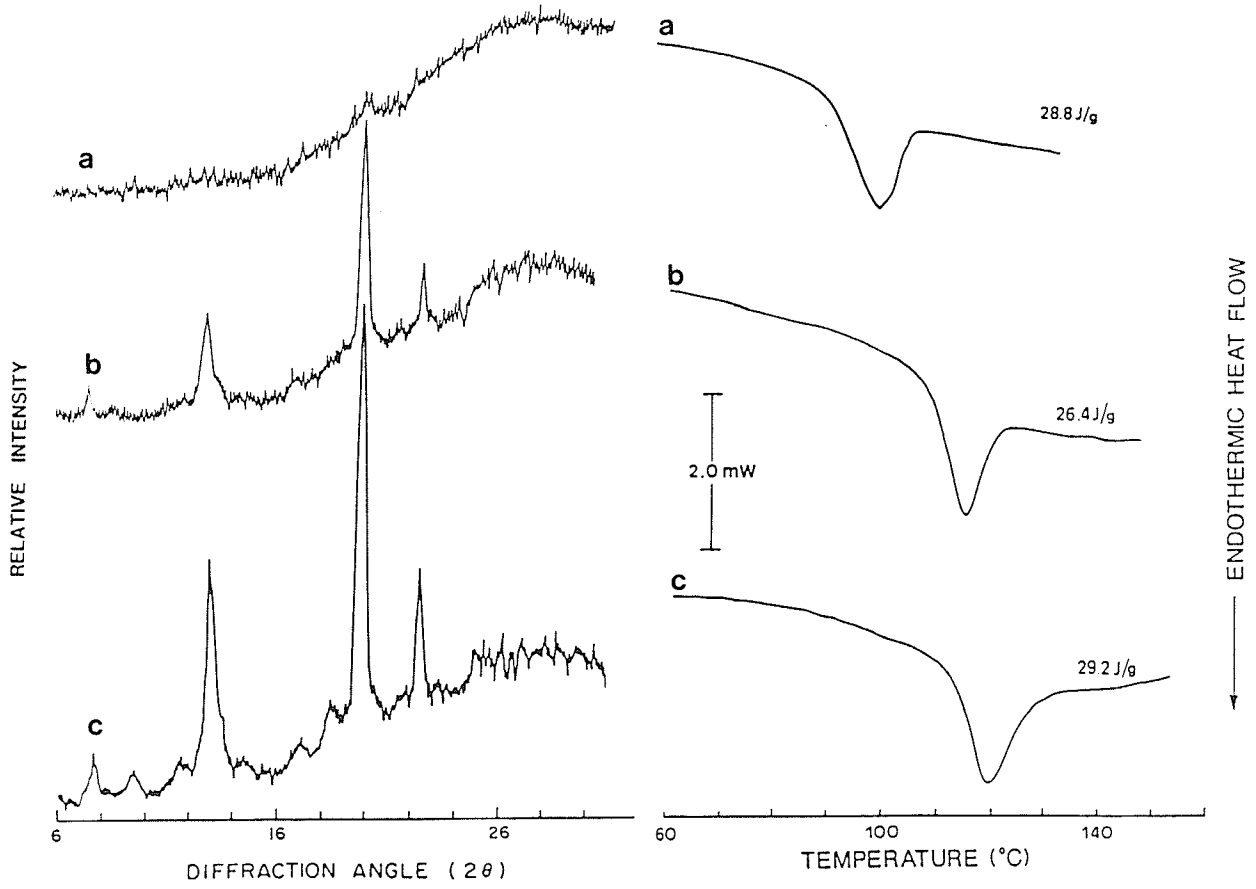

Gdn · HCl
Urea
TRANSITION ENTHALPY (J/g)
CONCENTRATION (M)
28
20
12
4
1.0
3.0
5.0
7.0
I
II

Figure 19. X-ray diffraction patterns (left) of dehydrated freeze-dried samples and DSC thermal curves (20% w/w complex in water); (a) form I; (b) form IIa; (c) form IIb.


a
b
c
RELATIVE INTENSITY
6
16
26
DIFFRACTION ANGLE (2θ)
28.8 J/g
26.4 J/g
29.2 J/g
2.0 mW
60
100
140
TEMPERATURE (°C)
ENDOTHERMIC HEAT FLOW

of magnitude (26.4-29.2 J/g). These results indicated that the major contribution to melting enthalpy of the complex is the enthalpy of helix→coil conformational transitions and thus differences in Tm for the various forms are of entropic origin; $Tm = \Delta H/\Delta S$.

Among the various salts examined, only cesium chloride exhibited mild dissociating effects with respect to the complex superstructure. This salt, however, stabilized the structure of form I(figure 20(a)): Tm increased with increasing molar concentration of CsCl (0.2-7.0m CsCl). On the other hand, CsCl seemed to cause dissociation of the supermolecular structure of forms IIa and IIb at molar concentrations above 4.0M and 5.0M, respectively, as evidenced by the corresponding thermal profiles (figure 20(b) and (c)). In fact, at intermediate salt concentrations (4.0-5.1m), double endotherms were observed that reflect the disordering process of forms I and IIa (or IIb). At much higher molar concentrations, single transitions were seen that correspond to melting of form I at equivalent salt concentration. These results, therefore, suggest that CsCl promotes disaggregation of amylose-lipid helices whereas it maintains their conformational order. This notion was subsequently corroborated by X-ray diffraction data (figure 21). With increasing concentration of CsCl, form II shows diminishing intensities in the characteristic V-diffraction lines (figure 21(b), (c) and (e)). For the 0.5 M-treated sample, after washing out the salt, there was a recovery of the three-dimensional order in the structure (form II), as seen in the pattern of figure 11(d); the washed sample was also found to have DSC transition characteristics (ΔH, Tm) indistinguishable from form II (data not shown).In contrast, exposing form II in a much stronger CsCl solution (6.0 M) yielded a structure that

Figure 20. DSC thermal curves of structural forms I (a), IIa (b), and IIb (c) of the complex (20% w/w) in cesium chloride solutions of varying molar concentrations. Mass of complex from top to bottom (mg): (left) 2.16, 2.08, 2.16, 2.14, 2.13, 2.09, 2.05, 2.33, 2.24, 2.06, and 1.99; (middle) 1.90, 2.07, 2.02, 1.91, 2.07, 2.03, 2.48, 1.92, 2.07, 2.14, and 2.13; (right) 2.27, 2.24, 2.34, 2.58, 2.08, 2.13, 2.08, 2.46, 2.15, 2.25, and 2.24. Heating rate 10^0C/min.

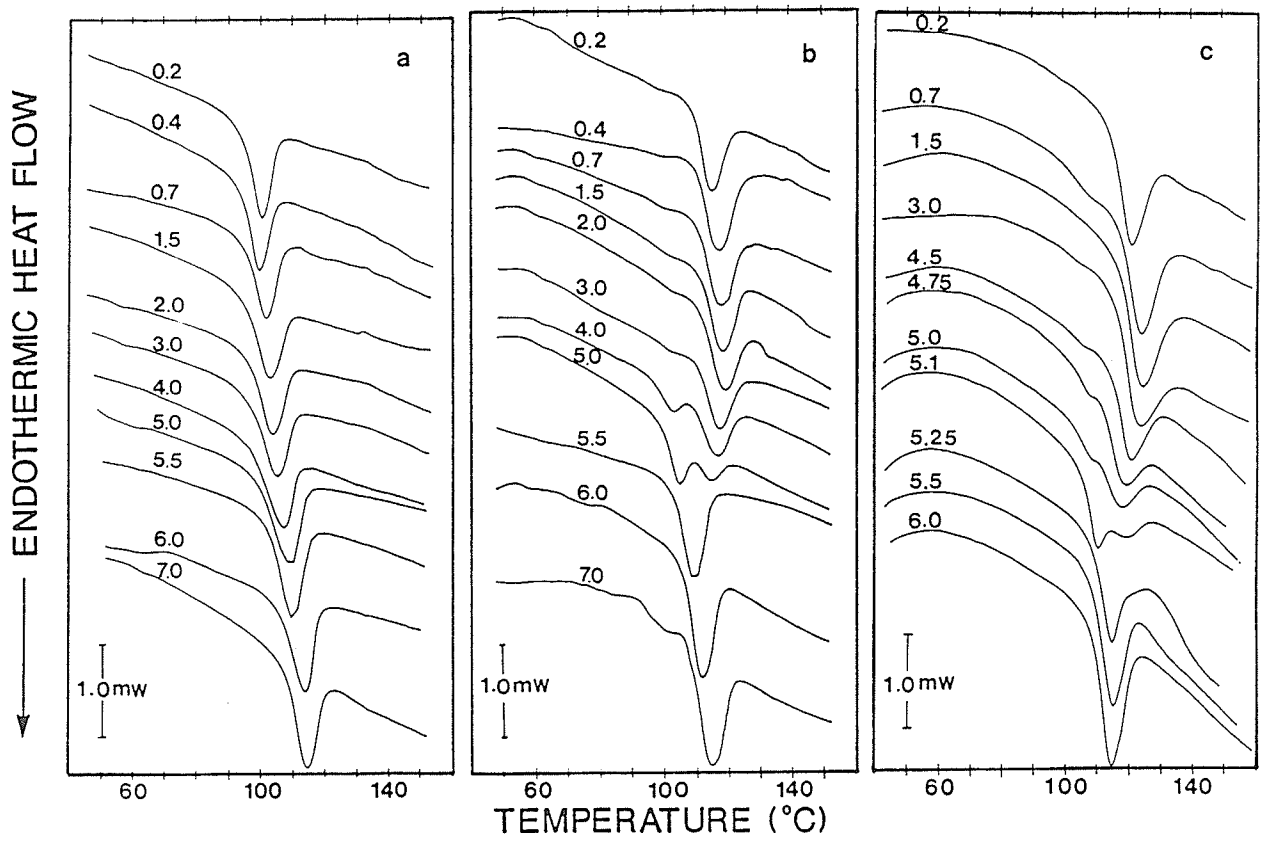
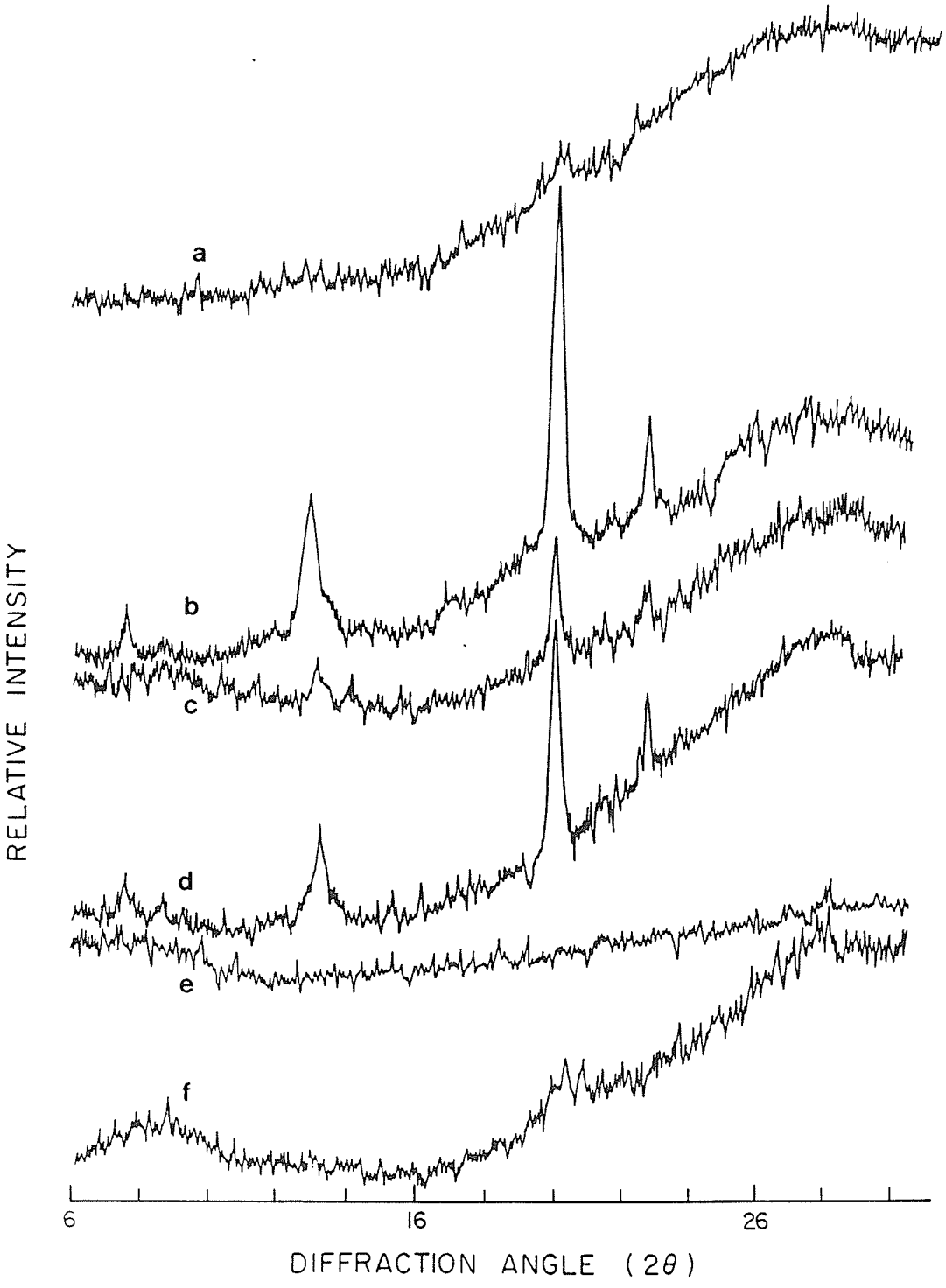
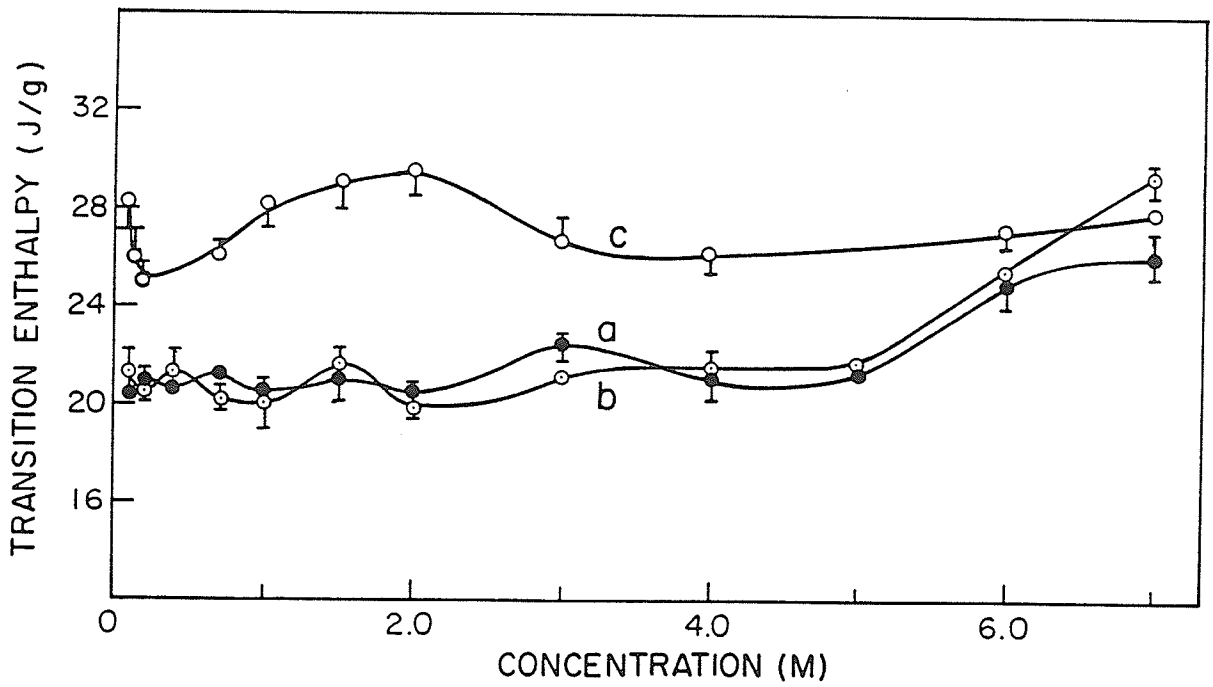

ENDOTHERMIC HEAT FLOW
a
0.2
0.4
0.7
1.5
2.0
3.0
4.0
5.0
5.5
6.0
7.0
1.0mw
b
0.2
0.4
0.7
1.5
2.0
3.0
4.0
5.0
5.5
6.0
7.0
1.0mw
c
0.2
0.7
1.5
3.0
4.5
4.75
5.0
5.1
5.25
5.5
6.0
1.0mw
60
100
140
TEMPERATURE (°C)

Figure 21. X-ray diffraction diagrams of wet glycerol monostearate-amylose complexes: (a) form I: (b) form II; (c) form II in 0.5 M CsCl; (d) samlpe c, washed; (e) form II in 6.0M CsCl; (f) sample e, washed.


a
b
c
d
e
f
RELATIVE INTENSITY
6
16
26
DIFFRACTION ANGLE (2θ)

had the characteristics of form I (X-ray; figure 21(b), (e) and (f) and DSC data) before and after thorough washing of the salt. Consequently, CsCl at high concentrations caused irreversible disruption of the partially crystalline supermolecular structure of the complex without altering, the conformation of individual helices. The transition enthalpies for all forms of the complex remained relatively constant (figure 22) over the entire concentration range (0.2-7.0 M CsCl), which provides additional evidence that the salt does not perturb the helical structure of the chains.

Figure 22. Transition entalpies of structural forms I (a), IIa (b), and IIb (c) of glycerol monosterate-amylose complexes with molar concentration of CsCl.


TRANSITION ENTHALPY (J/g)
32
28
24
20
16
0
2.0
4.0
6.0
CONCENTRATION (M)
a
b
c

MANUSCRIPT II

SOLUTE EFFECTS ON THE THERMAL STABILITY OF GLYCEROL MONOSTEARATE-AMYLOSE SUPERSTRUCTURES

INTRODUCTION

The stability of macromolecular structures is a sensitive function of their solvent environment (von Hippel and Schleich 1969). Following the pioneering work of Hofmeister, it is well established that neutral salts regulate order-disorder transitions and association-dissociation equilibria of biopolymers in solution (proteins, nucleic acids etc). In this context, there is a characteristic ranking of ionic effectiveness in promoting stability of macromolecular conformation known as the lyotropic or Hofmeister series. Despite a considerable amount of work devoted to this subject, there has been little progress in understanding the underlying phenomena of the lyotropic series. It is also known that these effects extend beyond the conformational behaviour of ordered macromolecules to salting out of non-electrolytes (small molecules), stability of lyotropic sols as well as surface tension and kinetics of chemical reactions in solution. In view of this generality, it would appear that such effects are a consequence of solvent structure modification by various ions. However, specific interactions between macromolecules and ions can occur which, in turn, modify solvent-polymer interactions and thereby the stability of the ordered structure. For example, conformational ordering and aggregation of charged polysaccharides (e.g. alginates, carrageenans) are sensitive to cation type (Morris *et al.* 1980; Norton *et al.* 1984). Furthermore, in considering aggregated structures, salt effects may be more complicated than those

affecting helix-coil transitions in solution. Intermolecular associations could influence the stability of ordered chains in the solid state and thereby modulate the sensitivity of a polymer matrix to solutions of electrolytes or other small molecular weight solutes.

The linear starch component, amylose, is precipitated from aqueous solutions of aliphatic compounds or iodine in the form of single-stranded helical complexes in which the guest molecule occupies the central cavity of the helix (Rundle and Edwards 1943; Mikus *et al.* 1946). X-ray powder diffraction of amylose complexes gives rise to a characteristic pattern, designated as V, which has different d-spacings from the other two polymorphs of amylose, A and B forms. The latter double-helical structures consist of more extended helices than the V-form; pitch height 21.04 and 20.80Å for A- and B-forms respectively, while 7.92-8.17Å for the V-forms (Horii *et al.* 1987; Hinrichs *et al.* 1987) depending on the bulkiness of the ligand molecule. On the basis of electron and X-ray diffraction data (Yamashita 1965; Zobel *et al.* 1967), a lamellar morphology has been proposed for the organization of helices in V-amylose; i.e. polysaccharide chains are folded and lie with their axes perpendicular to the crystal surfaces. From the Bragg equation, the calculated long spacings were 75-100Å (Manley 1964; Yamashita 1965), while the enzyme structural analysis data of Jane and Robyt 1984 suggested a lamellar thickness of ~ 100Å.

Recent calorimetric studies on phase transition behaviour of amylose-lipid complexes (Biliaderis and Galloway 1989; Galloway *et al.* 1989) have indicated that V-amylose helices exist in various states of aggregation depending on the thermomechanical history of the sample. Using saturated monoglycerides as complexing ligands, two thermally distinct

forms (I and II) were identified and characterized by X-ray diffraction, calorimetry and enzymic structural analysis methods. A morphological model for these metastable forms was also suggested (Biliaderis and Galloway 1989). Form I (low Tm) was obtained under conditions favouring rapid nucleation and was morphologically described as an aggregated state where ordered polymer chains are distributed randomly; i.e. in the hydrated state this form gives an amorphous X-ray pattern. In contrast, form II (high Tm) appeared as a polycrystalline aggregate with well developed long range order giving the typical reflections of V-diffraction pattern. Our observations were recently confirmed by Whittam *et al.* (1989) for complexes of amylose with linear alcohols (4-8 carbon atoms). Crystallization and annealing studies for amylose-monoglyceride complexes, under controlled temperature-time storage protocols (Biliaderis and Galloway 1989), further suggested that structural forms I and II belong to two distinct free energy domains that are separated from each other by high energy barriers; i.e. conversion of the kinetically preferred form I of the complex to the thermodynamically favoured structure II (state of low free energy) occurs only after partial melting of the former. Understanding the supermolecular structure, stability and transformations between the various forms is of considerable importance since it is known that the functional properties of starch containing foods are strongly influenced by the complexation of amylose with lipids during thermal processing. Our recent observations that sucrose stabilizes both forms of glycerol monostearate-amylose complex (manuscript I), while CsCl at high concentrations causes chain dissociation in form II without affecting the conformation of individual helices, prompted us to examine the effect of

various solutes on the thermal stability of the complex. In this communication, we report on the interactions between water and V-amylose superstructure as affected by the addition of neutral salts of the lyotropic series and the homologous glucose-based oligosaccharides (G_1-G_7).

Materials and Methods

Materials

Amylose, from potato starch with a DP of 1150 ([η] in 1N KOH= 156 $mL.g^{-1}$), was obtained from Aldrich Chemical Co. (Milwaukee, WI), while glyceryl monostearate was a product of Sigma Chemical Co. (St Louis, MO). Salts of ACS grade (CH_3COONa, NaCl, NaF, NaI, $NaNO_3$, Na_2SO_4, NaSCN, NH_4Cl, $CaCl_2$, LiCl, KCl and $MgCl_2$) were supplied by Fisher Scientific Co., while glucose and glucose-based oligosaccharides (G_2-G_7) were products of Boehringer Mannheim, Canada Ltd. (Dorval, Quebec).

Preparation of glyceryl monostearate-amylose complexes

The conditions for preparation of complexes were according to Biliaderis and Galloway 1989: amylose concentration 0.25% (w/v), amylose to ligand (glyceryl monostearate) ratio 5:1, isothermal crystallization at 60°C (Form I) and 90°C (Form II). Complexes were washed repeatedly with $CHCl_3$ to remove the free ligand, as assessed by DSC. Samples used for X-ray analysis were kept in the hydrated state, while those intended for DSC studies were freeze dried.

X-ray diffraction analysis

Wet amylose-V complexes were deposited as 1mm thick films on aluminium holders and analyzed with a Philips PW 1710 powder diffractometer equipped with a graphite monochromator: copper K_a radiation , voltage 40kV, sampling interval 0.45, scan speed 0.1x $2\theta°/$ s.

Differential scanning calorimetry

The DSC studies were carried out using a 9900 Thermal Analyzer equipped with a DuPont 910 cell base and a pressure DSC cell. The system was calibrated with indium (Biliaderis *et al*. 1985) and was operated under pressure by purging N_2 into the cell (1400 kPa). All measurements were carried out at 20% (w/v) solids in aqueous solutions of electrolytes and at a heating rate of 10°C/min. Under these conditions melting proceeds without reorganization of the metastable structures during heating (Biliaderis *et al*. 1985) (i.e. zero-entropy production melting). For the homologous glucose-based oligosaccharide series, DSC was carried out at two different weight ratios of complex: sugar: water (2.0: 1.6: 6.4 and 1.0: 2.0: 2.0). Data were collected at 0.4-s intervals and analyzed by the DuPont software analysis programs; reported transition enthalpies (J/g) and peak melting temperatures are means of triplicate analyses. Statistical differences between various solvent environments were determined, by Analysis of Variance, in conjunction with a Duncan's Multiple range test, and a paired t-test.

RESULTS

The DSC thermal curves (Figure 23) of glyceryl monostearate-amylose complexes grown isothermally at 60^0 and 90^0C, indicated that forms I (Tm 99.4 ± 0.3^0) and II (Tm 116.6 ± 0.2^0) were homogeneous preparations. The respective X-ray diffraction patterns of the two superstructures indicated that only form II has the three major reflection peaks of V-crystals at 7.36, 13.1 and 20.1 $2\theta°$. In contrast, an amorphous pattern was shown for form I. These results are consistent with the view that only Form II has sufficiently developed long range order in its structure, typical of a partially crystalline polymer (Biliaderis and Galloway 1989).

The effect of neutral salts, with Na^+ as the sole counterion present, on the melting temperature of the complex superstructures is shown in Figure 24 as a function of solute concentration. The relative ranking of the anions (at molar concentrations of salt <1.0) in stabilizing both structural forms was:

$$SCN^- < I^- < NO_3^- < F^- < Cl^- < CH_3COO^- < SO_4^{2-}$$

This order followed closely the classical Hofmeister series that is operative for stabilizing/destabilizing macromolecules as diverse in structure and conformation as DNA, proteins and synthetic polymers in aqueous solutions (von Hippel and Schleich 1969). Thus, SO_4^{2-} is clearly an effective stabilizer, while SCN^- and I^- destabilize the structure of forms I and II, decreasing the Tm considerably; the elevation or depression of Tm was a linear function of salt concentration. Other salts (NaCl, $NaNO_3$, NaF) affected Tm rather little over the entire range of salt

Figure 23. DSC thermal curves (20% w/w complex in water) and X-ray diffraction patterns of wet glycerol monostearate-amylose complex superstructures (forms I and II).

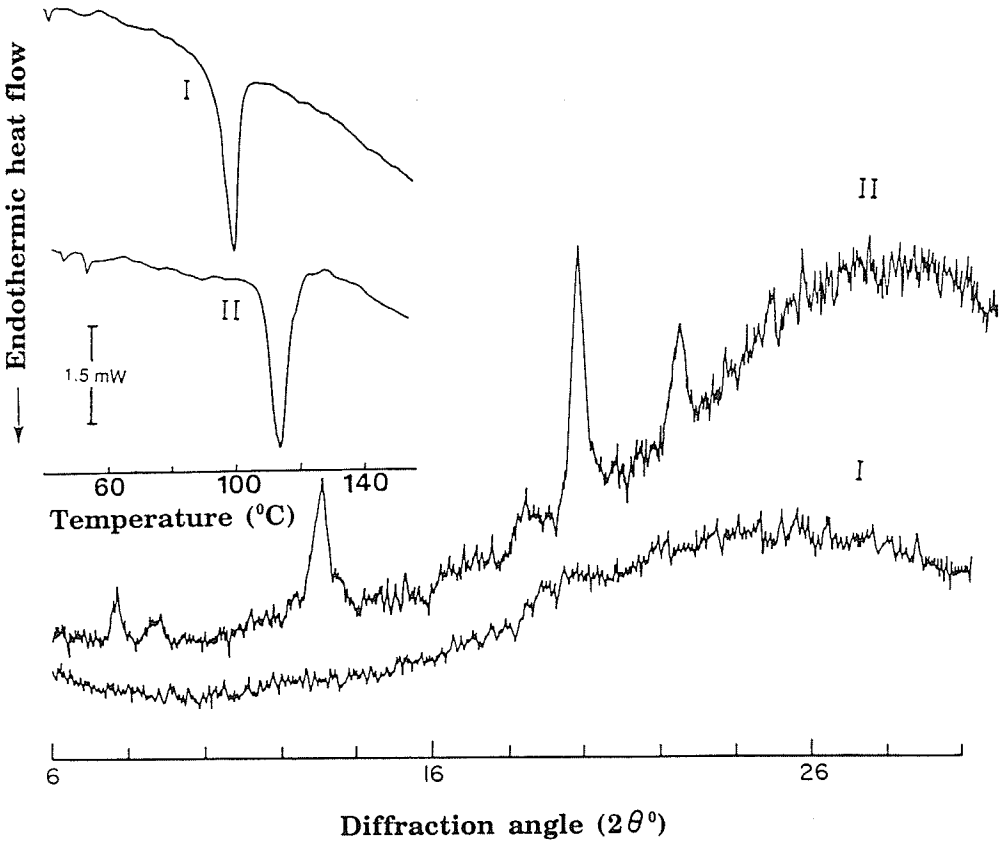
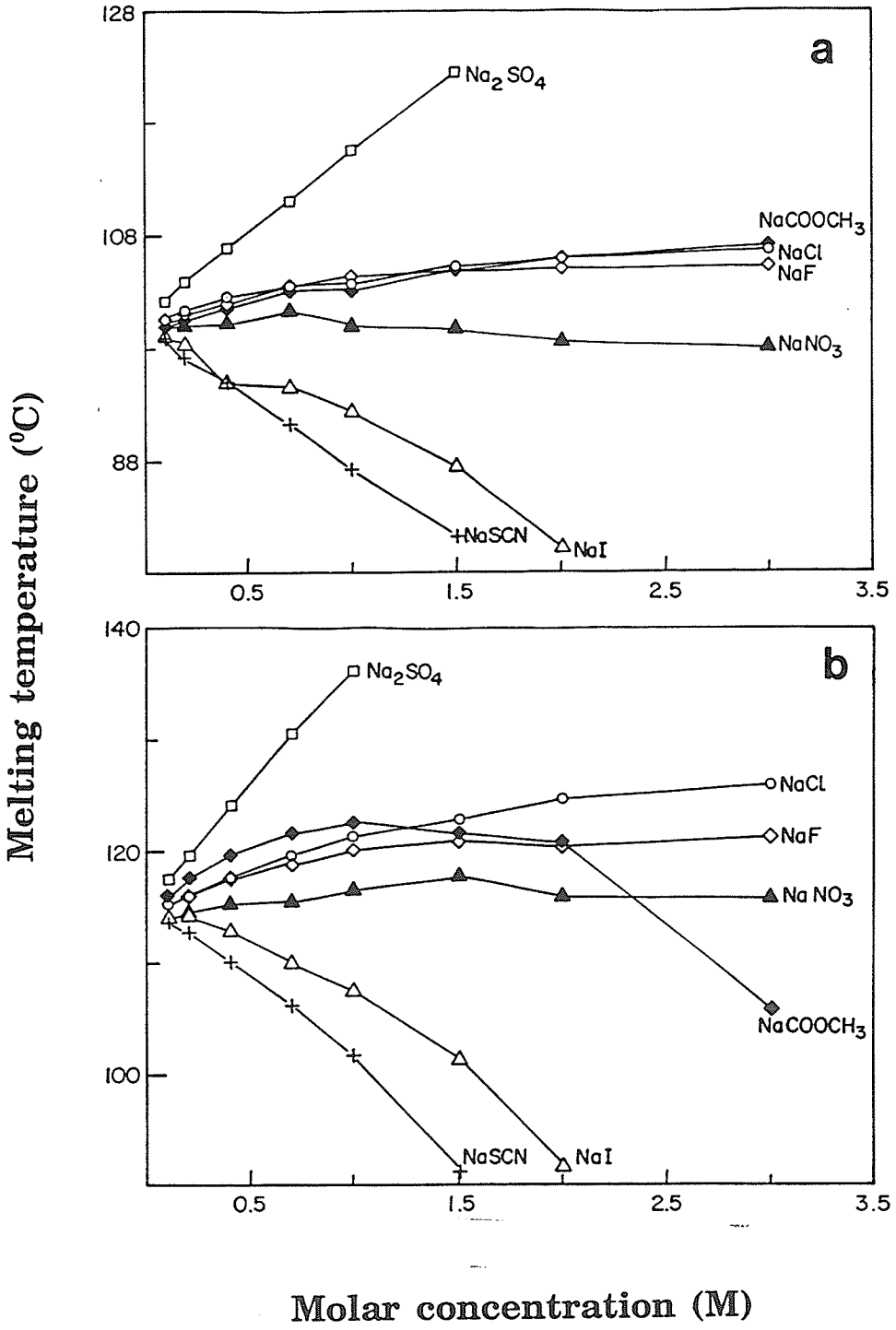

Endothermic heat flow
I
II
1.5 mW
60
100
140
Temperature (°C)
II
I
6
16
26
Diffraction angle (2θ°)

Figure 24. Changes in the melting temperature (Tm) of glycerol monostearate- amylose complexes (form I(a) and II(b)) as a function of molar concentration(M) of various sodium salts.


128
108
88
a
Na_2SO_4
$NaCOOCH_3$
NaCl
NaF
$NaNO_3$
NaSCN
NaI
0.5
1.5
2.5
3.5
140
120
100
b
Na_2SO_4
NaCl
NaF
$NaNO_3$
$NaCOOCH_3$
NaSCN
NaI
0.5
1.5
2.5
3.5
Melting temperature (°C)
Molar concentration (M)

concentrations tested. For I^- and SCN^-, in addition to effects on Tm, these ions markedly decreased the magnitude of the transition enthalpy (Table 2). At concentrations greater than 2.0M, NaSCN and NaI completely disrupted the ordered domains of both forms of the complex as evidenced by the lack of an endothermic transition.

To verify that the conformational responses and therefore the DSC transition data (ΔH, Tm), as described above, were not substantially influenced by the time of exposure of the complex superstructure to a particular ionic environment, the time dependence of the thermal properties of forms I and II were examined using the most potent destabilizers. Table 3 summarizes the values of the transition parameters for complexes equilibrated with varying NaSCN concentration solutions, for periods of 5 min and 24 hr, prior to DSC analysis. In general, concentrations up to 1.5M did not result in significant differences in ΔH and Tm between the two storage regimes. Similar observations were made for complexes exposed to NaI, where no differences were observed up to 2.0M concentrations (data not shown). These results imply that conformational disordering of V-amylose, is essentially independent of exposure time to the solvent at 25^0C and, instead, occurs mainly upon dynamic heating during DSC analysis.

The thermal responses of forms I and II to the series of anions tested exhibited similar trends at low salt concentrations (Figure 24). However, at high concentrations, anions which fell into the middle of the lyotropic series (NaCl, NaF and $NaCOOCH_3$) exerted different effects (relative magnitude and direction) on the complex superstructures. Thus, while the Tm-concentration relationships were clustered in the case of

TABLE 2. Transition enthalpies (ΔH,J/g) of glycerol monostearate-amylose complexes (forms I and II) as a function of molar concentration (M) of various sodium salts[a].

Concentration(M)	CH_3COONa	NaCl	NaF	NaI	$NaNO_3$	Na_2SO_4	NaSCN
				FORM I			
0.1	21.6±0.3a	20.3±0.1a	20.3±0.2a	20.7±0.2a	20.7±0.7a	23.7±0.7a	19.8±0.4a
0.2	22.4±0.1a	21.6±0.4a	20.3±0.4a	19.9±0.1ab	21.4±0.9a	21.7±0.7a	17.4±0.5b
0.4	20.1±0.2ab	21.1±0.4a	22.9±0.1e	18.3±0.1c	20.2±0.2a	21.6±0.1a	17.0±0.3b
0.7	20.6±0.1b	20.7±0.1a	20.4±0.2e	18.5±0.3c	23.8±3.4bc	21.6±0.2a	16.5±0.2c
1.0	20.7±0.2b	19.5±0.7a	21.6±0.1de	16.8±0.8d	19.7±0.1a	NM	14.8±0.4d
1.5	21.3±0.8ab	19.8±0.3a	21.7±0.2e	14.6±0.2e	20.1±1.2a	NM	9.9±0.9e
2.0	23.1±1.3b	21.4±0.5a	20.6±0.1ab	8.8±0.9f	18.8±0.9a	NM	4.6±1.1f
3.0	26.7±0.4c	19.8±0.3a	21.4±0.1bc	-	18.9±0.2a	NM	-
				FORM II			
0.1	21.5±1.3a	22.4±0.1a	21.4±2.1a	21.3±0.2a	24.3±0.4a	22.1±0.1a	20.4±0.4a
0.2	22.9±0.2a	22.4±0.3a	22.9±0.1a	20.2±1.1ab	19.65±0.1a	21.9±3.5a	19.7±0.1ab
0.4	23.9±0.9a	21.4±0.2a	22.3±0.1a	19.3±1.2b	18.4±0.1a	21.9±1.1a	19.0±0.7b
0.7	21.3±0.7a	23.5±1.3a	24.2±0.4a	17.0±0.1b	18.4±1.6a	22.8±0.4a	13.6±0.3c
1.0	23.5±0.1a	23.6±0.6a	23.0±0.9a	15.5±0.1c	21.2±2.0a	NM	10.9±0.3d
1.5	22.1±1.1a	22.9±0.1a	22.4±0.4a	10.1±0.5d	18.1±0.6a	NM	4.2±0.6e
2.0	20.8±0.5a	21.6±1.3a	22.6±0.1a	4.7±0.3e	19.2±1.7a	NM	3.5±0.5f
3.0	23.8±0.6a	21.7±1.6a	22.5±0.9a	-	17.7±0.8a	NM	-

[a] Column values followed by the same letter are not significantly different ($P \leq 0.01$) as determined by the Duncan's Multiple Range Test; NM values not reported due to non-equilibrium melting.

TABLE 3. Time-dependent effects on the thermal properties of glycerol monostearate-amylose complexes (forms I and II) in the presence of NaSCN[a].

	Form I				Form II			
Concentration (M)	Tm (°C)		ΔH (J/g)		Tm (°C)		ΔH (J/g)	
	5 (min)	24 (hr)	5 (min)	24 (hr)	5 (min)	24 (hr)	5 (min)	24(hr)
0.4	95.3±0.1a	95.4±0.3a	19.5±0.3a	19.6±0.3a	110.1±0.1a	109.9±0.1a	19.5±0.1a	18.3±0.1a
1.0	87.3±0.1a	87.9±0.4a	14.3±0.1a	13.4±0.1a	101.6±0.5a	101.1±0.4a	10.9±0.2a	10.7±0.4a
1.5	82.8±0.1a	84.3±0.1b	9.0±0.2a	4.8±0.5b	91.2±0.5a	93.8±0.4a	3.9±0.5a	2.2±0.2a
2.0	72.9±0.1a	71.2±0.1b	3.6±0.5a	2.0±0.2a	-	-	-	-

[a] Data followed by the same letter for each pair of values (5min vs. 24hr) are not significantly different ($P \leq 0.001$), as determined by a paired t-test.

form I, the respective plots for form II were quite spread. It is also interesting to contrast the responses in Tm with molar concentration of CH_3COONa between the two forms; acetate stabilized form I over the entire concentration range, while it depressed Tm of form II at concentrations above 2.0M. It would appear from these data that certain anions act differently at various levels of structural organization of amylose-lipid complexes.

In the case of aggregated states which consist of ordered polymer chains (e.g. amylose-lipid complexes) neutral salts and other perturbants are expected to affect structural order at two levels; one, the interchain association-dissociation processes (supermolecular level) and the other, helix-coil transitions (molecular level). Figure 25 presents the thermal responses of both forms of the glyceryl monostearate-amylose complex to varying molar concentration of NaI. With increasing concentration of this salt, there was a progressive decrease in Tm and enthalpy as well as a broadening of the endotherm. There was no indication that a multistep pathway is involved in the order→disorder transition of the structural domains of forms I and II. Under the experimental conditions employed in these studies (heating rate, solids/solvent ratio, electrolyte concentration), dissociation of aggregated helices and their conformational disordering seem to take place as one step thermal event. At 2.0M NaI, the low temperature (53-57^{0}C) endotherm corresponds to unbound monoglyceride, liberated upon disruption of helices.

Cations also exerted stabilizing effects on both complexes, as evidenced by the positive relationships between Tm and salt concentration (Figure 26), using Cl^- as the common counterion. The ranking of the cations

Figure 25. DSC thermal curves of structural forms I (a) and II(b); (20% w/w) in NaI solutions of various molar concentrations (0.2-2.0M). Mass of complex from top to bottom(mg): (a) 2.05(control), 2.14, 2.13, 2.05 and 2.30; (b) 2.09(control), 2.07, 1.99, 1.92 and 2.03. Heating rate 10^0 C/min.

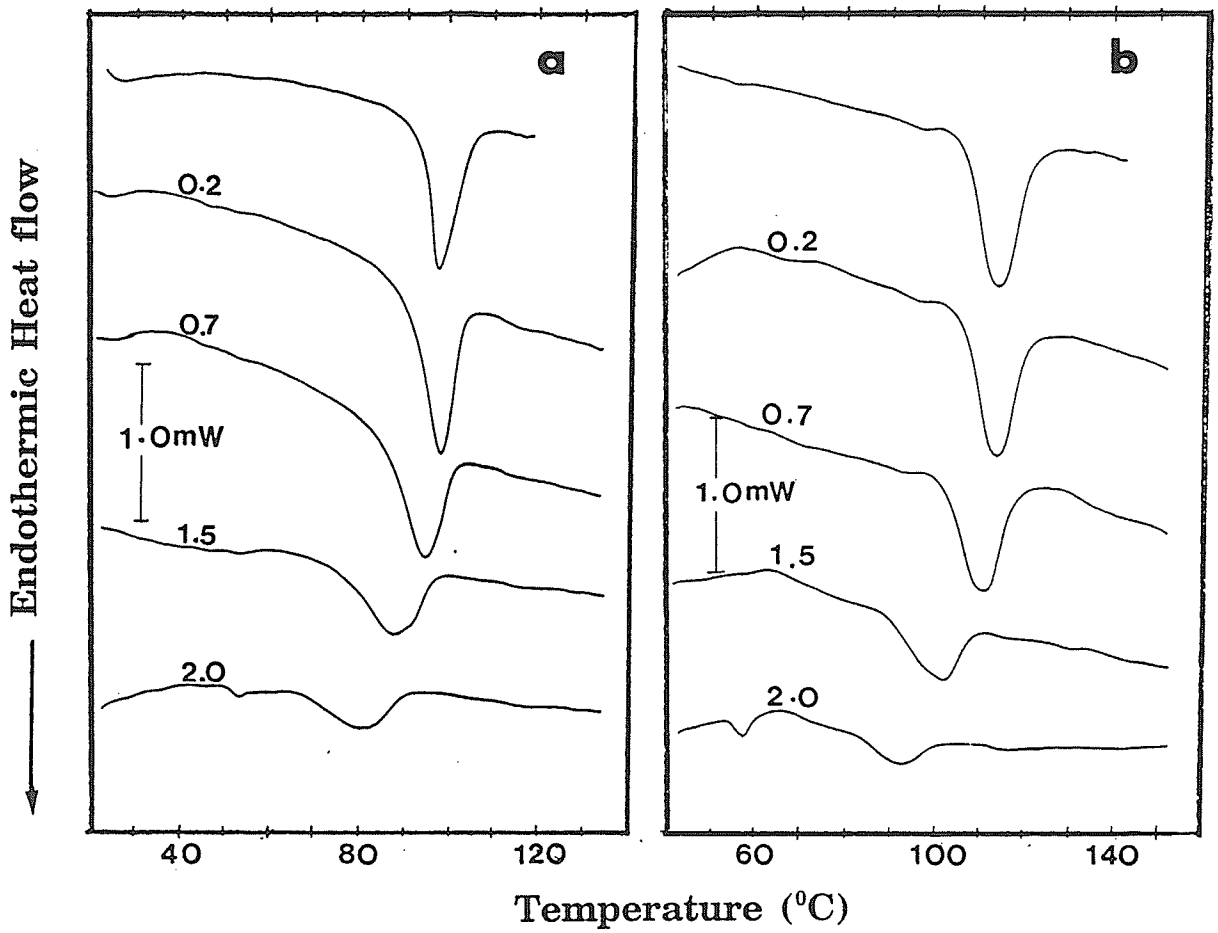
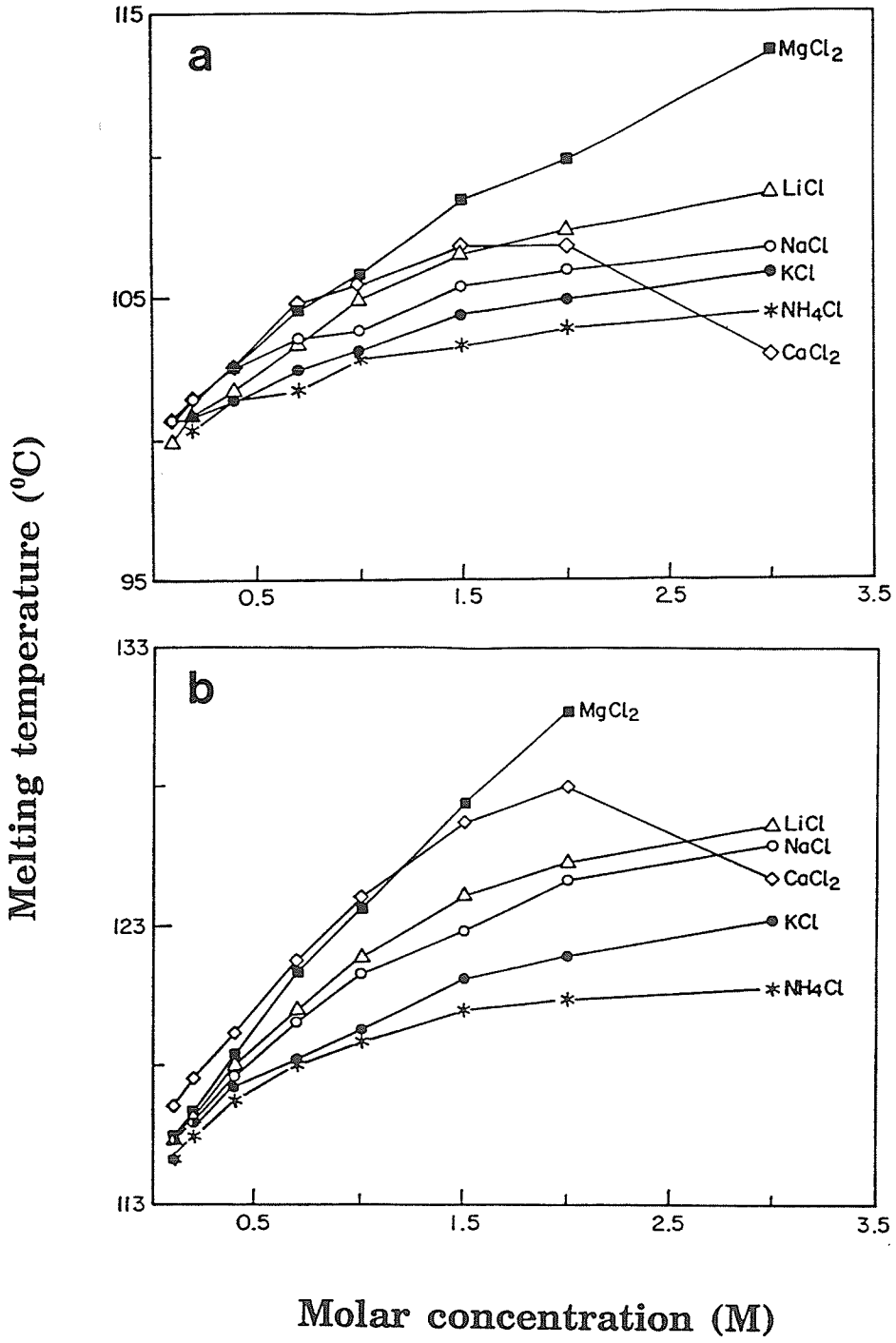

a
b
0.2
0.7
1.5
2.0
1.0mW
1.0mW
Endothermic Heat flow
40
80
120
60
100
140
Temperature (°C)

Figure 26. Changes in the melting temperature (Tm) of glycerol monostearate-amylose complexes, form I(a) and II(b), as a function of molar concentration(M) of various salts with chloride as common counterion.


a
b
MgCl2
LiCl
NaCl
KCl
NH4Cl
CaCl2
115
105
95
133
123
113
0.5
1.5
2.5
3.5
Melting temperature (°C)
Molar concentration (M)

in terms of their ability to stabilize the complex at low concentrations (<1.0M) was:

$$NH_4^+ < K^+ < Na^+ < Li^+ \leq Ca^{2+} \leq Mg^{2+},$$

a sequence that follows the Hofmeister series for cations. Values of Tm for the range of salts examined suggest that cations, differ less than anions in their molar effectiveness of stabilizing the complex superstructures. For $CaCl_2$, the Tm decreased markedly with increasing concentration at concentrations above 2.0M. Furthermore, form I exhibited higher sensitivity (dissociation) in the presence of 2.0M $CaCl_2$ than did form II.

The ΔH values of thermal dissociation of forms I and II remained relatively unchanged with the concentration of all neutral salts (Tables 2 and 4), except for NaI and NaSCN. Although minor differences in ΔH were observed (at $p \leq 0.01$) among certain concentrations for some of the electrolytes, the enthalpy values did not display any specific trends. It should be also noted that enthalpy estimates for transitions at >1.0M $NaSO_4$ were hampered due to non-equilibrium melting (Table 2), i.e. after partial melting, the complexes underwent reorganization during thermal analysis.

Glucose and the homologous series of maltooligosaccharides stabilized the complexes at both concentrations (e.g. Form I, Figure 27); the effects were greater for the high ratio of sugar to water (Figure 27b). These results agree with the findings of earlier reports (Bean and Yamazaki 1978; Wootton and Bamunuarachchi 1980; Ghiasi *et al.* 1982), which showed that addition of sugar to starches increases their pasting and gelatinization temperatures. One consequence of this behaviour is

Figure 27. DSC thermal curves of glycerol monostearate-amylose complex (form I) at complex to sugar to water weight ratios of 2.0:1.6:6.4(a) and 1.0:2.0:2.0(b). G_1-G_7 denote the glucose residues in the oligosaccharide. Mass of complex form top to bottom (mg): (a) 2.05(control), 2.30, 2.32, 2.20, 2.09, 2.11, 2.13 and 2.12; (b) 2.05(control), 1.94, 1.97, 1.88, 1.91, 2.05, 1.86, 1.96. Heating rate 10^0C/min.

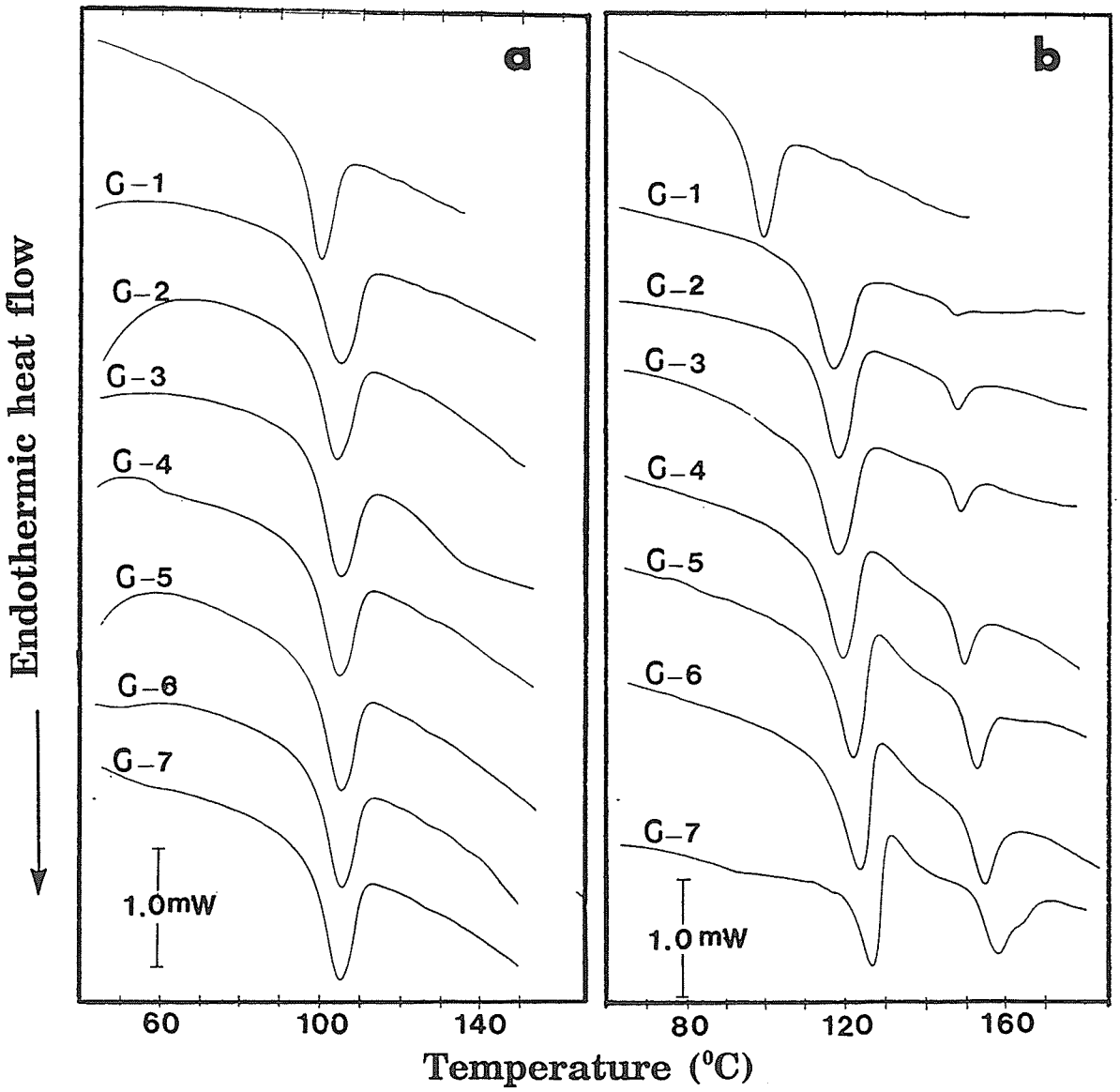

a
b
G-1
G-2
G-3
G-4
G-5
G-6
G-7
1.0mW
1.0 mW
Endothermic heat flow
60
100
140
80
120
160
Temperature (°C)

TABLE 4. Transition enthalpies (ΔH,J/g) of glycerol monostearate-amylose complexes (forms I and II) as a function of molar concentration (M) of various salts with chloride as common counterion[a].

Concentration (M)	NH_4Cl	$CaCl_2$	LiCl	$MgCl_2$	KCl
			FORM I		
0.1	21.3±0.2a	21.7±0.1ab	20.8±0.1a	20.0±0.3a	20.4±1.2a
0.2	21.2±0.1a	21.6±0.3ab	19.7±0.4a	20.3±0.1ab	20.2±1.0a
0.4	20.6±0.1b	18.4±1.2bc	20.9±0.8a	20.6±0.4ab	21.7±0.2a
0.7	20.5±0.2b	20.1±0.1ac	20.4±0.1a	20.4±0.1abcd	21.4±0.2a
1.0	20.6±0.3a	20.1±0.4a	18.2±0.7a	21.5±0.1bc	21.2±0.3a
1.5	20.5±0.1ab	20.5±0.2a	19.1±0.3a	21.3±0.1bc	21.2±0.6a
2.0	20.5±0.3b	20.0±0.9ab	19.7±0.8a	21.9±0.1c	20.5±0.1a
3.0	17.2±0.2c	21.6±0.1a	21.5±0.2a	23.1±1.4d	18.9±1.9a
			FORM II		
0.1	22.8±1.6a	21.8±0.1ab	21.9±0.3a	22.1±0.3a	22.3±0.1a
0.2	22.7±0.6a	21.8±0.2ab	21.9±1.3a	23.7±0.6bc	21.7±1.2a
0.4	21.9±0.6a	22.5±2.9b	22.1±0.3a	23.3±0.3c	21.7±0.5a
0.7	21.8±0.6a	21.9±0.1ab	21.3±1.6a	23.4±0.3bc	21.0±0.4a
1.0	22.1±0.1a	21.9±0.9ab	21.9±1.2a	23.5±0.3bc	22.4±0.1a
1.5	22.3±0.1a	22.9±0.6b	24.4±0.1a	24.3±0.3c	21.1±0.4a
2.0	22.4±0.4a	23.2±0.6b	23.2±1.0a	24.0±0.1dc	21.8±0.8a
3.0	22.1±0.1a	17.5±0.4a	22.5±0.2a	23.6±0.1dc	21.1±0.3a

[a] Column values followed by the same letter are not significantly different ($P \leq 0.01$) as determined by the Duncan's Multiple Range Test.

inhibition of starch gelatinization and possibly restrictive effects on the conformational disordering of starch molecules during heating of high sugar content food formulations. At the high oligosaccharide-to-water ratio (1:1), the DSC thermal profiles provided strong evidence of metastable melting (Figure 27b). Thus, following melting of the original structure a second high temperature endotherm was observed when sugars were included. The magnitude of this transition increased with increasing molecular weight of the oligosaccharide. The well-defined exothermic effect, between the two endotherms for G_5, G_6, and G_7, is indicative of structural rearrangement of form I during heating in the calorimeter. This behaviour is typical of non-equilibrium macromolecular crystals and has been previously reported for metastable V-amylose structures heated in the presence of limited amounts of water (Biliaderis *et al.* 1985; Biliaderis *et al.* 1986). Non-equilibrium melting was also apparent, although to a lesser extent, for form II at 1:2:2 mixtures of complex:sugar:water (data not shown). Furthermore, there was a slight reduction in the apparent transition enthalpy with increasing molecular weight of malto-oligosaccharide which suggests that the sugars restricted the extent of conformational disordering of helices upon heating; e.g. in the presence of G_1 and G_7, the respective values were 22.1 and 20.3 $J.g^{-1}$ for form I, and 23.8 and 20.2 $J.g^{-1}$ for form II.

DISCUSSION

The molecular and structural features of V-amylose with respect to chain conformation and unit cell dimensions have been revealed by a large number of X-ray studies (Rundle and Edwards 1943; Mikus *et al.* 1946; Zobel *et al.* 1967; Zaslow *et al.* 1974). The main structural motifs of this polymorph are helical chain segments stabilized by Van der Waals forces, hydrophobic interactions, and hydrogen bonding (between adjacent anhydroglucose residues O-2---O-3′, and interturn H-bonds O-2---O-6 and O-3---O-6) (Hinrichs *et al.* 1987). However, despite the similarity in chain conformation a detailed morphological description of V-amylose in the solid state, particularly as it forms under dynamic conditions of hydrothermal and mechanical processing of food materials, is difficult since organization of helices with varying degree of randomness can yield different supermolecular structures. Chain packing (positional and orientational) thus becomes an important determinant of local structural order and is expected to have an imprint on various macroscopic properties of this polymer. From the DSC and X-ray diffraction data of the present report and previous studies (Biliaderis and Galloway 1989), we infer that there is a relationship between thermal stability and structure of the two identified superstructures of amylose-monoglyceride complexes, forms I and II. While the thermodynamic and kinetic arguments for the existence of these forms have been presented in detail elsewhere (Biliaderis and Galloway 1989), most of the structural data point to differences in the degree of localized order. Form II has sufficiently developed long-range

order, typical of a partially crystalline structure and detectable by both DSC and X-ray diffraction techniques. On the other hand, form I seem to consist of structural domains of short-range order which, although not detectable by X-ray due to their size and/or imperfections, do respond thermally in the calorimeter. The latter structures represent a molecular organization between those of a crystalline state and a "truly" amorphous (glassy) state. Additional evidence supporting the suggestion of localized order in form I was obtained by freeze-drying the wet complexes, where the reflection lines at $2\theta^0$ 13.1 and 20.1 began to be visible; displacement and alignment of chains upon freezing improves the three-dimensional order of the aggregated helices without affecting their DSC thermal responses.

Changes in solvent quality by addition of neutral salts affected the conformational stability of both complex superstructures. In general, the effects of salts on the properties of glyceryl monostearate-amylose complexes were a strong function of the ionic species present (type and concentration) and followed the lyotropic series. With Na^+ as common counterion, "chaotropic" co-anions of high Hofmeister number, such as I^- and SCN^-, destabilized the structure (Tm was markedly shifted toward lower values and transition enthalpy was reduced with increasing salt concentration), whereas anions of low Hofmeister number, such as SO_4^{2-}, enhanced the stability of V-complex superstructures. Although the phenomenology of lyotropic series on biopolymer conformational stability is known, the origin of these effects still remains obscure (von Hippel and Wong 1964; von Hippel and Wong 1985). There are indications, however, that these ubiquitous effects are a manifestation of changes in water structure (von Hippel and Schleich 1969; von Hippel and Wong 1964) and

that preferential binding of ions on macromolecules, as frequently reported in protein literature (Sawyer and Puckridge 1973; Arakawa and Timasheff 1982; Arakawa and Timasheff 1984), may modulate the nature and balance of intra- and inter-molecular forces responsible for the stability of ordered states as well as the preferential hydration of the macromolecule. According to the work of Timasheff and co-workers (Arakawa and Timasheff 1982; Arakawa and Timasheff 1984) on proteins, stabilizing or salting-out effects on macromolecules are characterized by a large preferential hydration of the polymer, whereas extensive binding of the solute to macromolecule is frequently observed for those agents having structural perturbing effects.

In general, the order of effectiveness of various anions and cations (<0.1M) in displacing the Tm of the order-disorder transition of forms I and II resembles those for the effect of ions on pasting, gelatinization, solubilization and retrogradation of starch (Erlander 1968). The destabilizing anions SCN^- and I^- progressively decreased the Tm and increased the breadth of the transition of both forms without any evidence for interconversion between the two aggregated states, as was found in the presence of CsCl (4.0-5.0M). Although most structural perturbants exerted similar effects on both supermolecular structures, sodium acetate at high molar concentration (>2.0M) exhibited destabilizing action on form II, while it stabilized form I. These findings suggested that certain ions may affect differently the forces which influence the association-dissociation of helices than those involved in helix stabilization and thereby alter the thermal stability of the complex depending on its chain organization in the aggregated state. In contrast to the stabilizing action of most

cations (Figure 26) throughout the range of salt concentrations examined, $CaCl_2$ beyond the dilute salt level (>2.0M) lowered the transition temperature of forms I and II. This salt is also known to cause disruption of starch granules at room temperature (2.5-3.0M) (Gough and Pybus 1973; Evans and Haisman 1982) and has proven a potent destabilizer of native protein conformations (von Hippel and Wong 1965; Arakawa and Timasheff 1984). At much higher concentrations (>5.0M), calcium chloride solutions increased again the transition temperature of the complexes (data not shown), in agreement with the findings of Evans and Haisman (1982) on wheat starch.

With the exception of NaSCN and NaI, the results of transition enthalpy of the complex in various solvent environments (Table 2, 4) indicated that there were no wide differences between the two superstructures and over a wide range of electrolyte concentrations examined (0.1-2.0M). The enthalpy values of forms I and II in water were 21.2±0.6 and 22.2±0.8 $J.g^{-1}$, respectively. The slightly higher transition enthalpy of form II was maintained for almost all ionic environments. The process of complex dissociation involves the disruption of various stabilizing forces operating at both molecular and supermolecular level by heating in an aqueous medium. Energy is therefore required to dissociate the aggregated chains (i.e. to overcome intermolecular H-bonding between adjacent helices) as well as to disrupt the ordered conformation of chains. From the enthalpy data presented in Tables I and II and in the light of the postulated structural morphologies of forms I and II (Biliaderis and Galloway 1989), it would appear that melting enthalpies of amylose-monoglyceride complexes represent mainly the energy

for helix→coil transitions and that contributions from chain dissociation are minimal. Recent calorimetric data of Whittam *et al* (1989) indicated higher enthalpy values for crystalline than "amorphous" complexes (1.58 vs. 0.90 $J.g^{-1}$). The differences were attributed to a substantial contribution from intermolecular interactions in the case of crystalline complexes. However, the reported values were at least one order of magnitude lower than those of our studies as well as of other workers (Raphaelides and Karkalas 1988; Spies and Hoseney 1982). Variations in estimated enthalpy values could arise from the different thermal analysis systems used and their calibration, the crystallization conditions (temperature, ligand type) and purity of amylose. Such magnitude of difference in ΔH needs to be resolved first before any conclusions can be made on the relative contributions of intra- and interchain interactions to the overall transition enthalpy.

Glucose and maltooligosaccharides (G_2-G_7) raised the transition temperatures of both V-complex superstructures (Figure 27). This is in accord with earlier calorimetric studies which indicated that sugars and polyhydroxy organic compounds elevate the gelatinization temperature of starch (Wootton and Bmunuarachchi 1980; Ghiasi *et al*. 1982; Evans and Haisman 1982; Spies and Hoseney 1982). Although the stabilizing effects of sugars on starch gelatinization are of considerable importance in processing of high sugar content bakery items, the exact mechanism by which these solutes influence the transition behaviour of starch is unclear. A number of different explanations have been advanced, including competition for water, lowering of water activity and interactions of sugars with the amorphous parts of the granule (Spies and Hoseney 1982;

Lund 1984). However, it has been difficult to rationalize all the results on the basis of a particular theory. In view of the partially crystalline character of granular starch, Lelievre (1976) has applied a thermodynamic treatment of the melting data of starch/solute/water ternary mixtures using an extended version of the Flory (1953) equation which relates the Tm of a polymer to the diluent concentration. Aside from the simplifying assumptions made with regard to the magnitude of interaction coefficients between the three components (polymer-solute-solvent), this theoretical framework is based on the assumption of equilibrium states and processes, which usually are not valid in the case of heated aqueous starch systems (see discussion below). A similar relationship between Tm, water activity and volume fraction of water in the granules derived by Evans and Haisman (1982) from the Flory theory also suffers the same drawback.

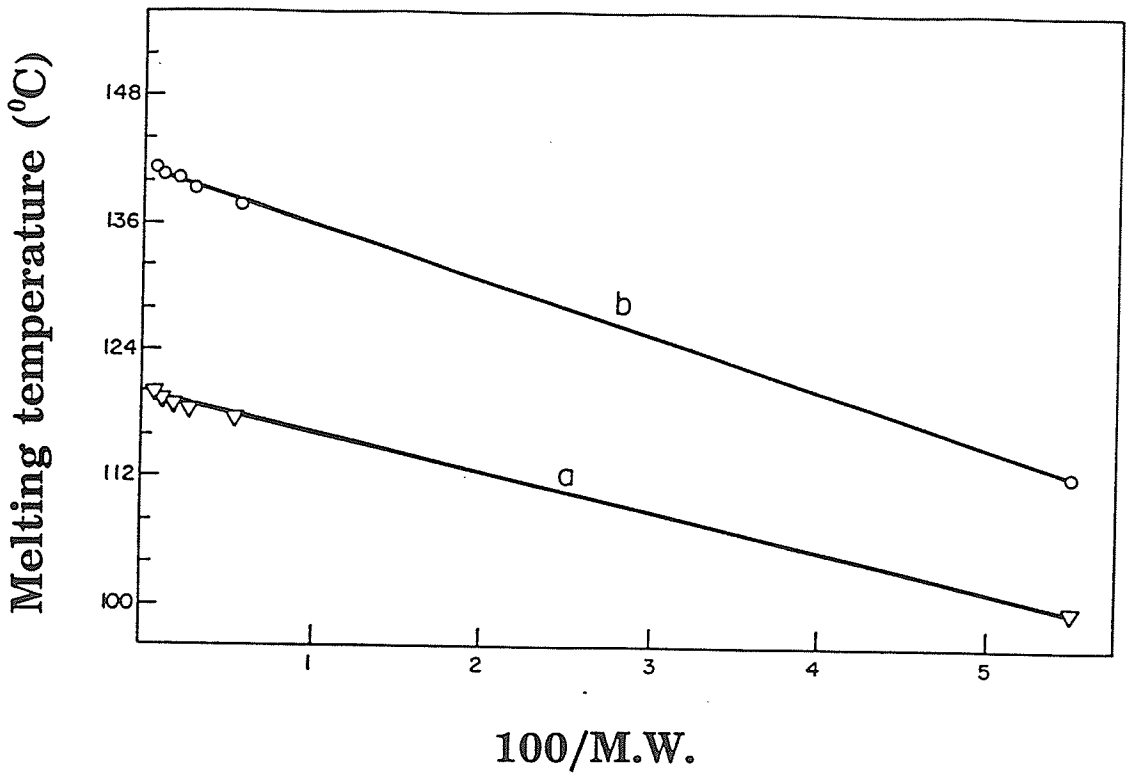
Following the theoretical analysis of van der Berg (1981), Slade and Levine (1985; 1987) have suggested that a glass-rubber transition precedes the melting endotherm of starch. This implies that melting is controlled by the mobility of the amorphous material surrounding the crystallites; i.e. melting can proceed only after exceeding the characteristic glass transition temperature (Tg) of the glassy regions of the granule. It has been also shown (Biliaderis *et al.* 1986; Zeleznak and Hoseney 1987) that Tg is highly sensitive to water/heat plasticization; i.e. Tg of dry starch is greatly depressed by small amounts of water (plasticizer). In this context, starch gelatinization was described as a non-equilibrium process since melting of crystallites is kinetically-constrained by the immobile glass at temperatures below Tg (Maurice *et al* 1985; Slade and Levine 1987; Bilideris *et al* 1986). Furthermore, within the Tm-Tg range, where

molecular mobility of the amorphous chain segments is enhanced, composite thermal effects due to partial melting of metastable crystallites (endothermic), reorganization (exothermic) and final melting of more stable crystallites have been reported for heated aqueous starch systems (Biliaderis *et al.* 1985; Biliaderis *et al.* 1986a,b). The thermal curves in Figure 27b clearly indicate that such events also occur for V-amylose complexes in the presence of low molecular weight carbohydrates. The reason why V-amylose superstructures undergo reorganization during heating lies in the metastable (non-equilibrium) nature of their ordered domains. Following partial melting of the less stable helices, reorganization is favoured since the remaining helical chain segments can act as nuclei and the temperature is still below the melting point of the equilibrium crystals. Reorganization and formation of regularly packed arrays of helices yield a state of much lower free energy (thermodynamically stable).

An alternative approach has been taken by Slade and Levine (1987) to explain the elevation of starch gelatinization by sugars. According to these workers, a water-sugar mixture is a plasticizing cosolvent which is less effective in depressing the Tg of starch relative to water alone (i.e. sugar-water exerts an antiplasticizing action). In view of the molecular weight dependence of Tg for polymeric materials (Billmeyer 1984), they further suggested that antiplasticization is enhanced by increasing the molecular weight and concentration of the solute. Their experimental data for the effects of homologous series of sugars, present as cosolvents, confirmed this prediction. As the MW of the cosolvent increases, the cosolvent becomes less efficient in suppressing the Tg of

granular starch and the Tm of crystallites is raised. Using these insights, we have tested their hypothesis for the glyceryl monostearate-amylose superstructures. As shown in Figure 28, the MW of the water-maltooligosaccharide cosolvents was indeed inversely related to the melting temperature of the complex. These results add further support to the argument that polyhydroxy compounds influence the melting of starch structures by elevating the Tg of the respective amorphous phase which in turn causes the melting events to commence at a higher temperature.

Figure 28. Transition temperature(Tm) as a function of 100/MW of cosolvent (water + glucose oligomer) for 3-component mixtures of glycerol monostearate-amylose : sugar : water (1.0:2.0:2.0 parts by weight): (a), form I; (b), form II.


Melting temperature (°C)
148
136
124
112
100
1
2
3
4
5
b
a
100/M.W.

MANUSCRIPT III

ACTION OF α-AMYLASES ON V-AMYLOSE SUPERSTRUCTURES

INTRODUCTION

The digestion of starch by α-amylases has been the subject of many investigations in recent years due to its analytical and possible nutritional significance in the dietary fiber concept (Dreher *et al.* 1984; Preiss and Levi 1980; Berry 1986; Ring *et al.* 1988). In general, these studies have revealed that susceptibility of starch to amylolytic enzymes, not only depends on the source of starch and enzyme, but also on the processing and storage conditions to which starch is subjected. For example, cooking greatly improves the digestibility of poorly-digestible starches (Dreher *et al.* 1984), presumably due to granular disorganization and changes in the crystallinity of starch materials. Furthermore, incompletely gelatinized products of whole wheat (as assessed by differential scanning calorimetry), exhibit reduced rates of α-amylolysis and thus elicit low glycemic responses (Holm *et al.* 1988; Holm 1988). Other factors known to affect the kinetics and extent of α-amylolysis of starch include amylose-amylopectin ratio and retrogradation of starch molecules (Dreher *et al.* 1984; Holm 1988; Sievert and Pomeranz 1989). Furthermore, since enzymic hydrolysis of starch-containing foods is a heterogeneous reaction, available surface area is an important parameter. The latter is related to granule and particle sizes (coarse vs finely ground materials), macrostructure (compact vs porous) and the presence of other constituents (e.g. cell walls, proteins) which may act as physical barriers (entrapment of starch) and thereby restrict enzyme accessibility

to starch. Depending on the severity of conditions thermal processing affects the physical state and form of starch and, therefore, can influence starch availability and glycemic responses; this was shown for extruded and drum-dried wheat flour products (Bjorck *et al.* 1984).

Complexation between lipids and amylose, which readily takes place during heat processing of starch, also appears to influence the susceptibility of starch to enzymic degradation (Mercier 1980; Schweizer *et al.* 1986). It has even been proposed that these complexes could be regarded as component of the dietary fiber (Larsson and Miezis 1979). The effects of lipid copmlexation on amylose digestibility was further examined by Holm *et al.* (1983). Although complexed amylose was highly resistant to α-amylase *in vitro*, compared to free amylose in solution, complete digestion of the complex was obtained when a large excess of enzyme was added. Complexes of amylose with lysolecithin were also found to be completely absorbed in the rat small intestine. Nevertheless, the plasma glucose and insulin levels, following ingestion of complexed amylose, were significantly lower than those after ingestion of free solubilized amylose. These results, therefore, imply a slower degradation of amylose-lipid complexes for both *in-vitro* and *in-vivo* situations. Earlier studies by Eliasson and Krog (1985), also demonstrated that complexes with long saturated monoglycerides are more resistant to enzymic breakdown than complexes with shorter aliphatic chains or greater degree of unsaturation for the ligand. Although these findings clearly indicated that complexation of amylose with monoacyl lipids is responsible for a greater resistance to enzymic hydrolysis, there have been no relationships established between α-amylase susceptibility and structure/physical

properties of amylose inclusion complexes (V-amylose). In two recent reports (Biliaderis and Galloway 1989; Galloway *et al.* 1989), several structurally distinct forms (I, IIa and IIb) for the amylose-saturated monoglyceride complexes in the aggregated structure were identified and characterized. According to the structural model put forward, form I, obtained under conditions of rapid nucleation, was morphologically described by a random distribution of helices being in little crystallographic register with one another. In contrast, the supermolecular structures of forms IIa and IIb were typical of partially crystalline state in which well-defined crystallites are embedded in disordered regions; differences between IIa and IIb mainly lie on the degree of crystallinity/perfection in the ordered domains of the complex. The gross morphological differences in structure and properties for such V-amylose superstructures as well as the ability to generate these forms under controlled conditions (temperature-ligand type-time) of complexation from dilute amylose solutions have prompted us to investigate the effects of chain organization in the solid state on the resistance of complexes to α-amylolysis. Furthermore, this study aimed to provide additional insights into the structure and properties of the various forms of amylose inclusion complexes, and the influence the superstructure might have on dietary fiber analysis data which are based on enzyme digestion of starch.

Materials and Methods

Materials

Potato starch amylose was obtained from Aldrich Chemical Company (Milwaukee, WI). The molecular characteristics of this polysaccharide were: $[\eta]$ in M KOH = 156 $ml.g^{-1}$, corresponding to a degree of polymerization 1150 (d.p.= 7.4 $[\eta]$), and iodine affinity 18.9g I_2/100g. Alpha-amylase (E.C. 3.2.1.1)from *Bacillus subtilis* and hog pancreas were purchased from Boehringer Mannheim Canada Ltd. (Dorval, Que.). The lyophilized *B. subtilis* preparation was reconstituted into a suspension (100mg/ 7ml) by addition of $(NH_4)_2SO_4$ solution (3.2M, pH 6.0). The enzyme from hog pancreas was supplied as suspension in this solution by the manufacturer. Enzyme activities for the α-amylase preparations were determined according to the method of Jane and Robyt (1984); one unit (IU) is defined as the quantity of enzyme required to produce a reducing potential of 1 μ mole glucose per min from a 0.5% (w/v) soluble starch (Fisher Scientific, Ottawa, Ont.) solution (0.003M NaCl, 0.05M phosphate buffer, pH 5.5 and 6.9 for *B. subtilis* and hog pancreas, respectively). Gel permeation chromatography equipment and gel media were products of Pharmacia Canada Ltd. (Dorval, Que.). All other reagent grade chemicals were obtained from Fisher Scientific Ltd. (Winnipeg, Man.).

Preparation of the complexes

The conditions for preparation of complexes were according to Biliaderis *et al.* (1985); amylose concentration 0.25% (w/v), amylose to ligand ratio 5:1, isothermal crystallization at 60°C (Form I) and 90°C (Form IIa) for 24 hr. Complexes were washed repeatedly with $CHCl_3$ to remove the free ligand. In addition to forms I and IIa, an annealed sample (IIb) of hydrated form IIa, prepared by isothermal annealing (30% w/w complex in water, 120°C/2h), was used in this study. Except for samples of IIa used in annealing experiments and all forms of the complexes used for turbidity measurements, the complexes were freeze dried. The freeze dried powders, prior to α-amylase digestion, were passed through a 0.5mm sieve to obtain complexes of similar particle size.

α-Amylase digestion

Complexes were digested with α-amylases using procedures similar to those of Jane and Robyt (1984). Two digestion studies were conducted. In the first study, hydrolysis was performed batchwise, and conditions involved suspending 500 mg of complexes in 60 ml of appropriate buffer,(6mM NaCl, 0.02% NaN_3, 0.1M phosphate buffer, pH 5.5 and 6.9 for *B.subtilis* and hog pancreas α-amylases, respectively). The complexes to enzyme activity ratio was 30mg/IU. The digestion mixtures were gently agitated using a mechanical shaker. At specified digestion periods (from

5hr to 72hr), each batch was centrifuged (8,000 ×g) the supernatant was filtered and total carbohydrate analysis was carried out using the phenol sulphuric method (Dubois *et al.* 1956); extent of hydrolysis was determined by expressing the total carbohydrates in the supernatant (as glucose) as percent of the initial complex. The enzyme digestion of the residue material was arrested by the addition of 10 volumes of 0.1M $HgCl_2$. After 24h the residues were centrifuged (8,000 ×g) and washed twice with distilled water. The samples intended for X-ray diffraction were kept in the hydrated form, while those intended for DSC and chromatography were freeze dried. In a second study, which was also performed batchwise, 750mg of complex (form IIa) was suspended in 90 ml of appropriate buffer. Depending on the digestion conditions, the complex to enzyme activity ratio was varied from 1IU to 20IU per 30mg of complex. At a specified digestion time (24, 48 and 72hr), sample aliquots (2ml) of the digest supernatants were withdrawn for total carbohydrate analysis (Dubois *et al.* 1956).

X-ray diffractometry

X-ray diffraction analysis of freeze-dried and rehydrated (30% H_2O) complexes was performed with a Philips PW 1710 diffractometer equipped with a graphite crystal monochromator: copper K_a radiation; voltage 40kV, sampling interval 0.45s; scan speed 0.1 $2\theta^0$/sec.

Differential scanning calorimetry

The DSC studies were carried out using a 9900 thermal analyzer equipped with a Dupont 910 cell base, and a pressure DSC cell (Biliaderis *et al.* 1985). All measurements were carried out at 20% (w/v) (unless otherwise specified) and at a heating rate of 10^0C/min. The lyophilized samples were kept at least for 2h to equilibrate with the solvent. Results presented are means of at least duplicate measurements.

Turbidity measurements

Changes in turbidity of aqueous suspensions (0.37mg/ml) of glycerol monostearate-amylose complex, used as an index of particle density, were monitored by transmittance readings at 580 nm at 25^0C.

Debranching of waxy maize starch with pullulanase

Amylopectin from waxy maize (35 mg) was dispersed in 1 ml H_2O and 1.5 ml hot Me_2SO. After diluting with acetate buffer (3.5 ml, 0.1M, pH 5.7), the solution was heated (85^0C for 15 min) to completely dissolve the polysaccharide. Debranching was carried out using 4 units (0.1 ml) of pullulanase at 37^0C for 6hr. The enzyme digest was subsequently heated in boiling water for 20 min to inactivate the enzyme and filtered prior to gel filtration chromatography.

Gel Chromatography

Gel permeation chromatography of the complexes and the enzyme-treated residues was performed on a Sepharose CL-6B column (2.5 x 96cm). Elution was carried out with 0.1 M NaOH solution at a flow rate of 40 ml/hr at 25^{0}C. The delipidized complexes (boiling in 85% methanol for 2hr), dissolved in the same solvent, were applied onto the column (10mg), and fractions of 5 ml were collected. Total and void volumes were determined using glucose and blue dextran, respectively. Also, the debranched amylopectin digest was used to further calibrate the column. Eluent fractions were analyzed for total carbohydrates by the phenol-sulphuric method (Dubois *et al.* 1956).

^{13}C CP/MAS NMR

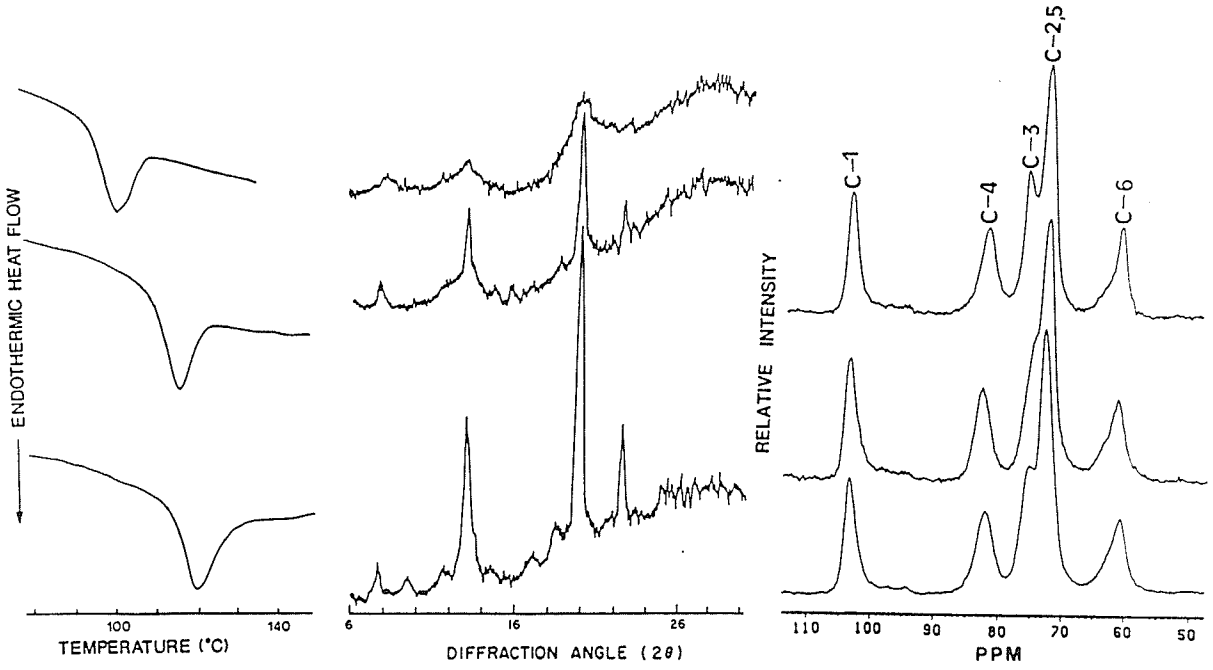
Solid-state CP/MAS carbon-13 spectra were obtained at 75.46 MHz on a Bruker CPX-300 spectrometer. Spinning rate of 4 kHz and spin locking and ^{1}H decoupling fields of ~320 kHz were used. Spectra are referenced to external Me_4Si. A contact time of 1 ms was used for all spectra with a recycle time of 2s. Other parameters were: line broadening 5Hz; acquisition time 34 msec.

RESULTS AND DISCUSSION

Structural considerations

A range of supermolecular structures for the amylose-monostearin complexes were prepared by altering the temperature of crystallization. Forms I, IIa and IIb differ in the organization of helices, as evidenced by their X-ray diffraction and DSC data of Figure 29. The thermal curves indicated that all complexes were pure preparations, yielding single endothermic transitions at 98.9±0.7^{0}C, 114.3±0.3^{0}C and 124.5±0.6^{0}C for forms I, IIa and IIb, respectively. The X-ray patterns of rehydrated (~30% H_2O) freeze-dried powders of these complexes confirmed our previous findings (Biliaderis and Galloway 1989) on the structure of these forms; i.e. while IIa and IIb gave patterns typical of partially crystalline states, form I shows limited degree of long range order in the aggregated state. For the latter, it is worth commenting here that it is only after freeze-drying and rehydration that form I develops a diffused V-type pattern, presumably due to slight modification of the structure via chain aggregation during freezing. This form, when isolated as a hydrated precipitate from dilute amylose solutions, gives rise to a completely amorphous X-ray pattern (Biliaderis and Galloway 1989). The relative intensities of the characteristic diffraction lines of V-crystals (at 7.36, 13.1 and 20.1 $2\theta^0$) were more pronounced in the order of I< IIa< IIb. The observed narrow range of melting enthalpies (20.5-26.7 J/g) among the three structures is also consistent with our earlier proposal (Biliaderis

Figure 29 . DSC thermal curves (20% w/w complex in water), X-ray diffraction patterns and ^{13}C CP/MAS NMR spectra of glycerol monostearate-amylose complexes: For each set of data, the patterns correspond to Form I top), Form IIa (middle) and Form IIb (bottom).


ENDOTHERMIC HEAT FLOW
100
140
TEMPERATURE (°C)
6
16
26
DIFFRACTION ANGLE (2θ)
C-1
C-4
C-3
C-2,5
C-6
RELATIVE INTENSITY
110
100
90
80
70
60
50
PPM

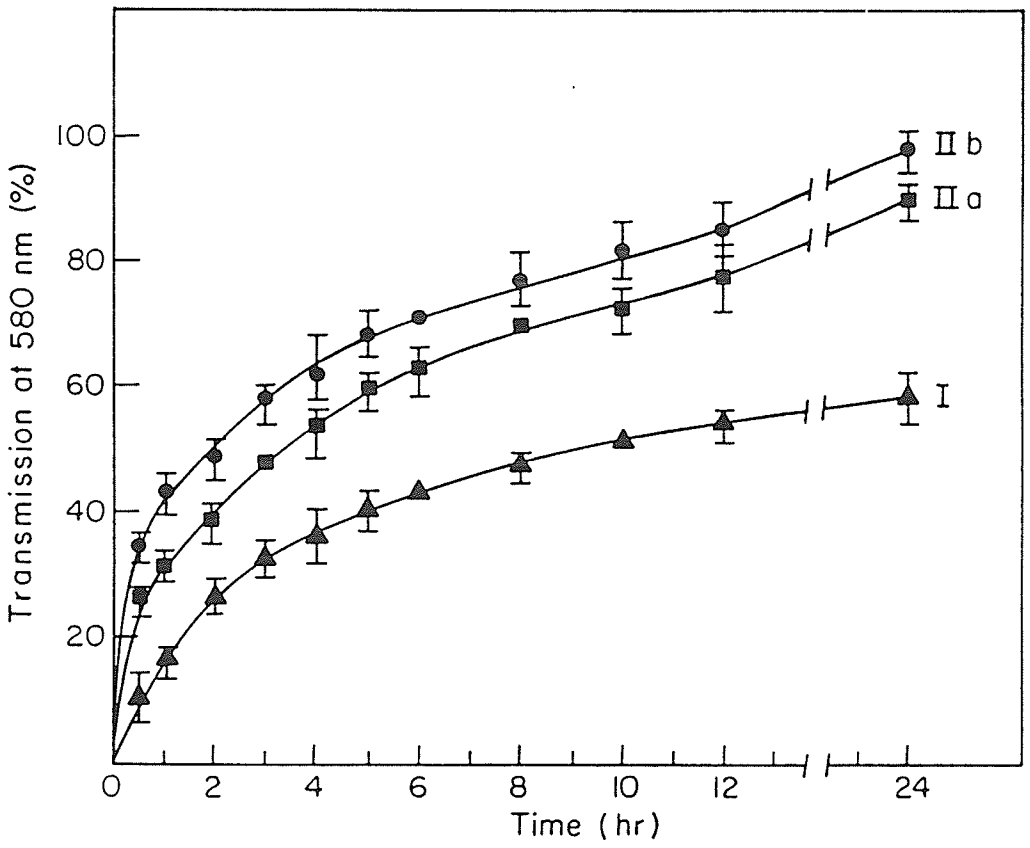
and Galloway 1989) that the enthalpy change on dissociation of amylose-lipid complexes represents mainly the energy required to disrupt individual helices and includes very little contributions from intermolecular effects. These results are in contrast to the recent findings of Whittam *et al.* (1989). Their DSC data on the complexes of amylose with short chain linear alcohols showed much greater values for the dissociation enthalpy of crystalline complexes (1.58 J/g) than that of "amorphous" forms (0.90 J/g). These researchers, therefore, suggested that a substantial portion of the transition enthalpy of crystalline V-complexes corresponds to the thermal energy needed to overcome the crystal lattice intermolecular forces. Such conclusions, however, must be seen with caution, since the reported range of enthalpy values (0.9-1.6 J/g) is at least one order of magnitude lower than those from previous studies (Raphaelides and Karkalas 1988) and the results of the present report.

The cross polarization and magic angle spinning (CP/MAS) ^{13}C NMR spectra of complexes (Figure 29) showed resolved resonances in the regions of 103, 82-83 and 61-62 ppm which, according to Gidley and Bociek (1985), may be assigned to C-1, C-4 and C-6 sites. Another signal at 75.8 ppm, partly resolved for forms I and IIb, corresponds to C-3 sites, while the resonance at 72-73 ppm, having much greater intensity than the other signals, was assigned to unresolved resonances of both C-2 and C-3 sites (Gidley and Bociek 1985). The chemical shifts for the glycerol monostearate-amylose complex are closely similar to those of other V-amyloses (Gidley and Bociek 1988) prepared with ligands known of inducing various polymorphic forms of the complex (V_6 and V_7). These results, therefore, support the findings of Gidley and Bociek (1988) that ^{13}C

chemical shifts are not sensitive to expansion of the amylose helix from a six-to a sevenfold repeat. Furthermore, despite a large variation in the long range order of the three forms of the complex (Figure 29), very little change in the ^{13}C chemical shifts was observed. Line widths in ^{13}C CP/MAS spectra of α-D-glucans and their oligomers are also known to be more sensitive to crystallinity changes than the chemical shifts (Gidley and Bociek 1985): broader signals were obtained in the order of crystalline cyclodextrins< ordered oligomers< ordered polymers< amorphous (gelatinized) starch. The ^{13}C NMR spectra of Figure 29 showed no structural variation in line width at half heights for the resolved resonances. It would seem reasonable, therefore, to conclude that increasing perfection of the complex superstructure is insufficient to bring about alterations in chemical shifts, line widths or resonance multiplicities for certain carbon sites. The ^{13}C CP/MAS spectra of V-amyloses are similar to the spectrum of amorphous lyophilized amylose (Gidley and Bociek 1985),except that the shape of C-1 line in the case of amorphous amylose is broader and has an upfield tailing. The similarities in C-1 and C-4 chemical shifts for single helical amylose structures and amorphous α-D-glucans have been interpreted as evidence for the presence of amylose/lipid inclusion complexes in the native granular starches (Gidley and Bociek 1988).

The time-dependent changes in turbidity of V-amylose complexes in aqueous suspensions are shown in Figure 30. Clearly, forms IIa and IIb exhibited more rapid particle sedimentation than form I. This implies a greater particle density for IIa and IIb and is in accord with the hypothesis that a more compact chain organization exists in the solid state of these forms (Biliaderis and Galloway 1989).

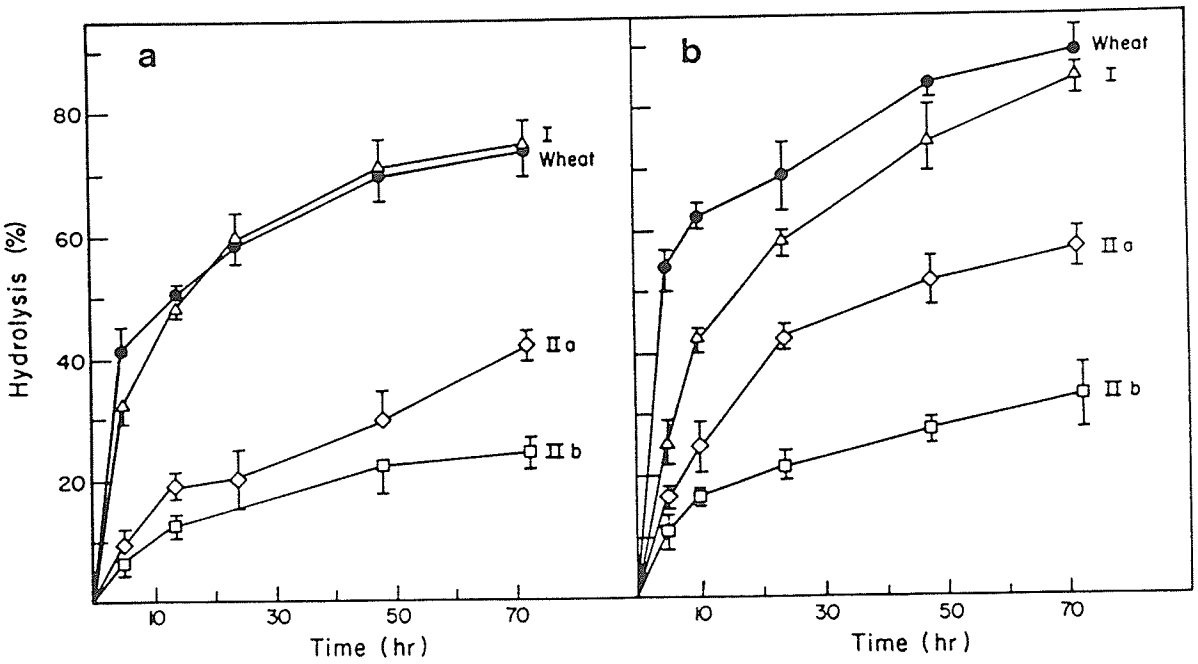
Figure 30. Time dependent changes in turbidity of glycerol monostearate-amylose complex suspensions (0.37 mg/ml): Form I(▲), Form IIa(■) and Form IIb(●).


Transmission at 580 nm (%)
100
80
60
40
20
0
2
4
6
8
10
12
24
Time (hr)
II b
II a
I

α-amylolysis of complexes

Hydrolysis of amylose-monostearin complexes with *B. subtilis* (Figure 31a) and hog pancreas (Figure 31b) α-amylases in heterogeneous reaction mixtures was performed to assess the effect of supermolecular structure on the *in vitro* digestibility of the complexes. Native granules of wheat starch were also included in these comparative kinetic studies. The hydrolysis profiles in Figure 31 indicate that after a rapid rise in soluble carbohydrates during the early stages of incubation, hydrolysis proceeds at a much slower rate later on. Hydrolysis action presumably occurs more rapidly in the disordered regions of the complexes, partly accounting for the biphasic reaction kinetics. Under the conditions employed in these studies, the degree of hydrolysis did not reach at constant plateau values even after 72hr of digestion for most samples. The rate and extent of degradation of the complexes with both enzymes decreased in the following order: form I> form IIa> form IIb. This ranking corroborates with the proposed structural features of these forms regarding the degree of organization and perfection of ordered domains of aggregated chains; i.e. form I with the less ordered structure (more open) in the solid state exhibits the highest susceptibility to α-amylase. Thus, supermolecular structure of V-complexes does affect the accessibility of enzymes to the solid substrate and thereby controls α-amylolysis kinetics. Interestingly, both enzymes at a concentration of 3.3 IU/100mg substrate hydrolyzed wheat starch granules more rapidly than the three forms of the complex.

Figure 31. Enzyme hydrolysis (0.5g solids/60ml buffer, 3.3IU/100mg substrate) of glycerol monostearate-amylose complexes by (a) *B. subtilis* and (b) hog pancreas α-amylases: Wheat starch(●), Form I(Δ), Form IIa(◊) and Form IIb(□).


a
b
Hydrolysis (%)
80
60
40
20
I
Wheat
IIa
IIb
10
30
50
70
Time (hr)

In view of the relative resistance to hydrolysis of glycerol-monostearate-amylose complexes, particularly of forms IIa and IIb, and the potential contribution of these materials to the amount of resistant starch, α-amylase digestion studies were performed at different initial enzyme concentrations (Table 5). Using form IIa in these experiments, the extent of hydrolysis increased with increasing level of α-amylase. At 62 IU/100 mg complex, form IIa was completely hydrolyzed to soluble oligosaccharides after 72hr of digestion. Consequently, even crystalline forms of the amylose complexes can be fully degraded under prolonged reaction time and high enzyme levels. Whether limiting hydrolysis yields are attained *in vivo* and thereby modulate the availability of V-amylose, it would depend on its supermolecular structure, as it forms during thermal processing of foods, the physical form of the overall food matrix, and the reaction time with the salivary and pancreatic α-amylases. In model studies it was observed that amylose-lysolecithin complexes are completely hydrolyzed and absorbed in the gastro-intestinal tract of rats (Holm *et al.* 1983). With regard to contributions to the dietary fiber values obtained for starch containing foods, the possibility of the complexes to resist the thermostable microbial α-amylase during the high temperature- high enzyme concentration conditions employed (Holm *et al.* 1983) seems unlikely. Similar responses to α-amylolysis with varying amounts of enzyme observed for form IIa (Table 5) have been reported for amylose, amylopectin and starch gels (Ring *et al.* 1988). Moreover, α-amylolysis of native starches shows increased hydrolysis rates and lower amounts of resistant residues with increasing level of enzymes (Ring *et al.* 1988; Colonna *et al.* 1988).

TABLE 5. Extent of hydrolysis of glycerol monostearate-amylose complex (form IIa) by α-amylase in a heterogeneous reaction mixture (0.75g solids/90ml buffer;37°C)

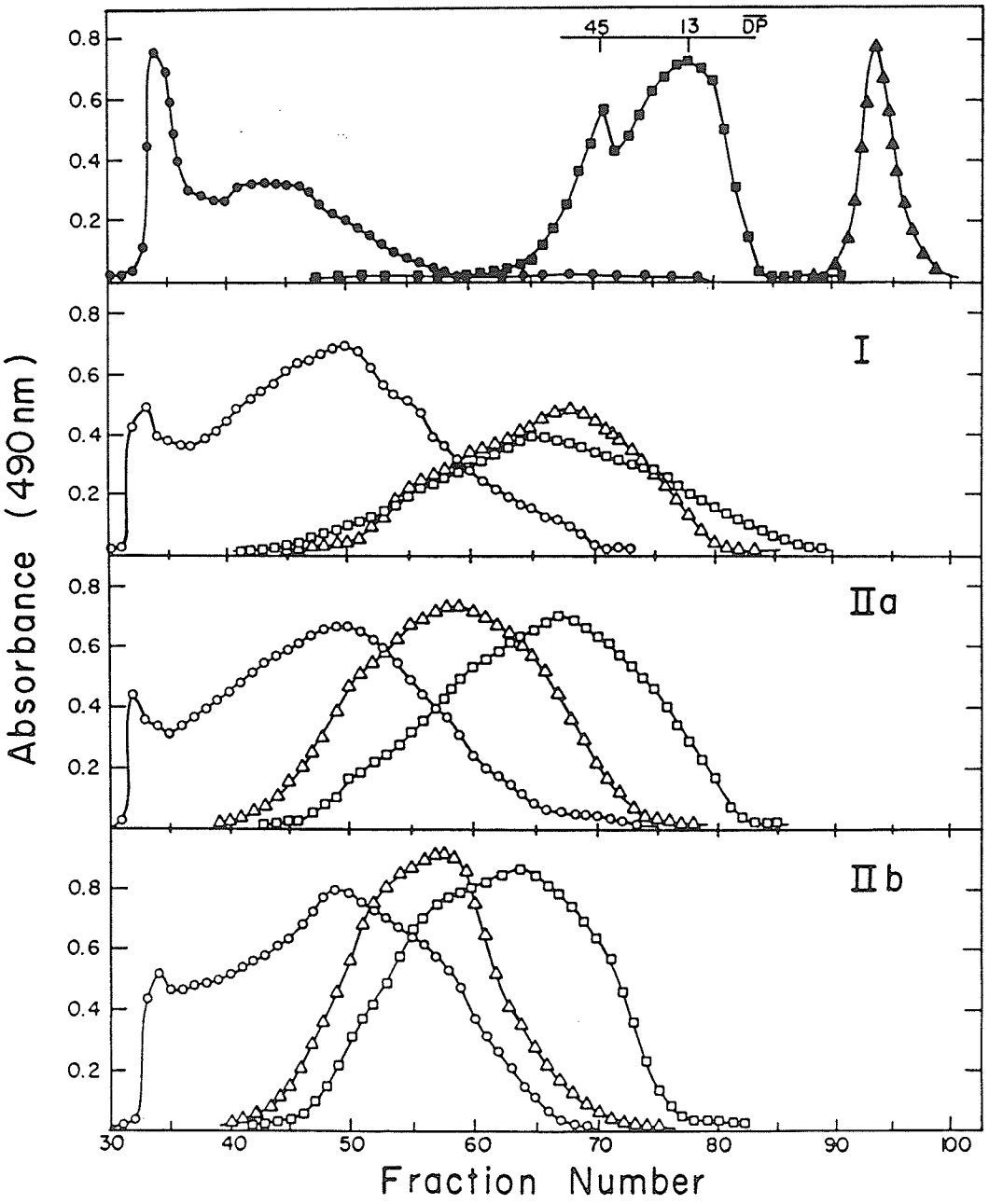
Enzyme concentration	*Bacillus subtilis* α-amylase(pH 5.5)			Hog pancreas α-amylase(pH 6.9)		
(IU/100mgComplex)	Time (hr)			Time (hr)		
	24	48	72	24	48	72
3.3	20.4±3.1[a]	26.7±1.7	37.3±6.1	37.9±2.8	44.3±5.6	49.5±3.1
16.3	29.8±2.3	33.8±6.4	39.7±1.7	44.6±1.9	47.1±3.8	56.1±3.2
33.0	39.6±2.4	54.9±1.2	70.3±2.3	55.8±3.3	80.8±1.6	83.4±5.8
66.0	79.9±7.6	93.6±2.7	95.3±2.1	91.1±4.3	99.1±4.3	100.2±3.6

[a] Means ± s.d (n=2)

The results of Figure 31 and Table 5 reveal that the *B. subtilis* α-amylase (BSA) gives a lower degree of hydrolysis than the hog pancreas enzyme (HPA) under identical conditions (enzyme concentration-time-complex form). These differences in hydrolysis rates are probably due to the greater number of substrate binding subsites of the BSA (9 glucose units) (Robyt and French 1963) compared with the HPA (5 glucose units) (Robyt and French 1967). As a result, the BSA may leave a greater number of undigested fragments and, therefore, exhibit lesser accessibility to short amylose chain segments in the aggregated structure.

Gel permeation chromatographic profiles (Sepharose CL-6B) of the delipidized residues of complexes following 48h digestion with 3.3 IU/100mg complex are presented in Figure 32. Extensive chain depolymerization was found in both enzyme digests even for the more resistant forms of the complex superstructures, IIa and IIb: the chain distributions of the resistant amylodextrin fragments were shifted toward lower molecular size than the distributions of native amylose. These elution patterns differ from those of enzymic residues of wheat starch granules after digestion with the BSA (Colonna *et al.* 1988). Even after extensive degradation (53-91% hydrolysis) with this enzyme, Collona *et al.* (1988) observed very little change in the chromatographic profiles of the resistant material, compared to native starch, on Sepharose CL-2B. On the basis of their findings, these researchers suggested that starch granules are not equally susceptible to hydrolysis by *B. subtilis* α-amylase and that the enzyme-resistant material consists mainly of starch granules with very little degradation; i.e. enzyme action is not uniform throughout the population of starch granules. In contrast, to granular starch, the

Figure 32. Chromatography on Sepharose CL-6B (2.5x 96cm, eluted with 0.1M NaOH, flow rate 40ml/hr, 25°C) of glycerol monostearate-amylose complexes: Native (○) and residues after hydrolysis (48h, 3.3IU/mg complex) with *B. subtilis* (△) and hog pancreas (□) α-amylases. Chromatograms of blue dextran(●), debranched waxy maize(■) and glucose(▲) are given at the top

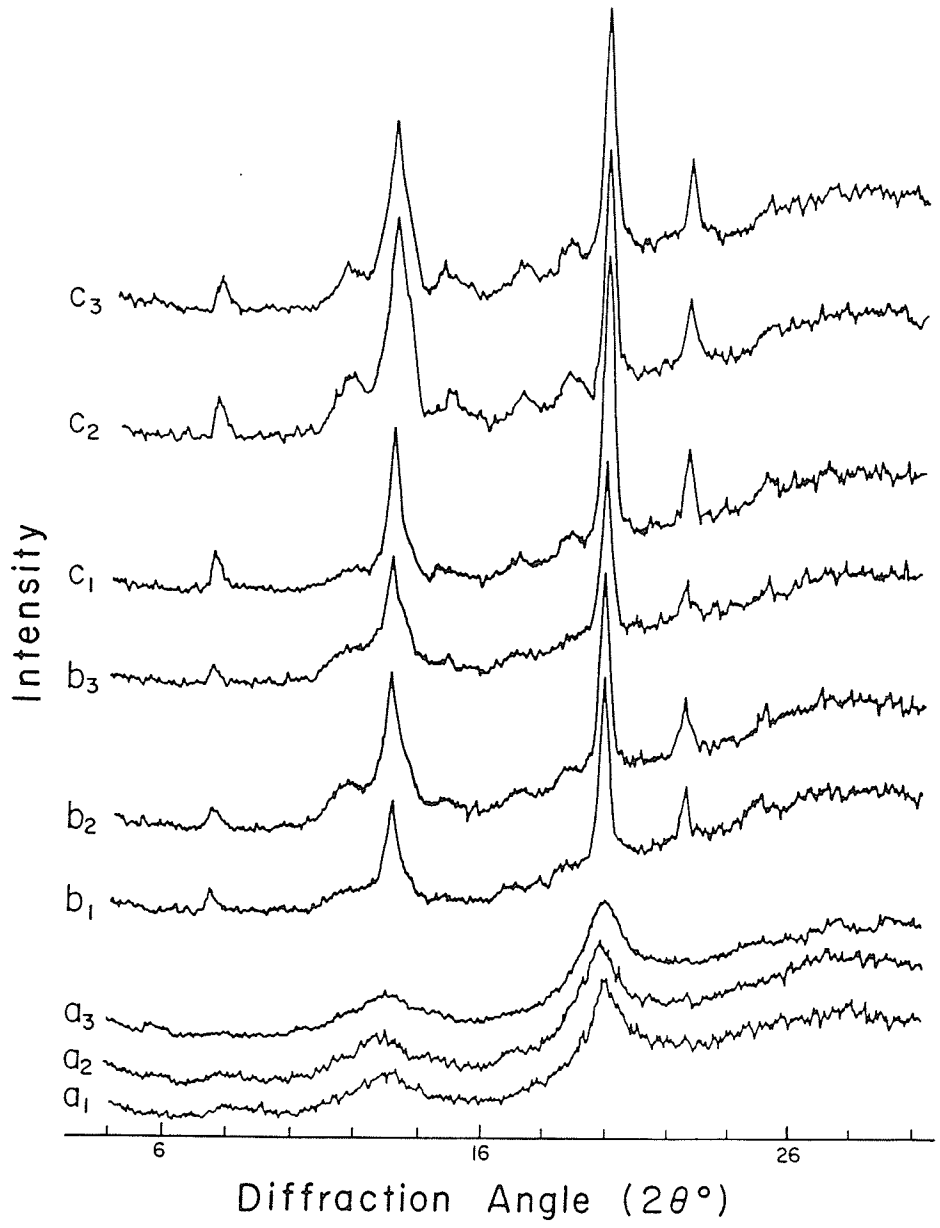

45
13
DP
I
IIa
IIb
Absorbance (490nm)
Fraction Number
0.2
0.4
0.6
0.8
30
40
50
60
70
80
90
100

results in Figure 32 do not support a similar mode of macromolecular degradation for the glycerol monostearate-amylose complexes. Even after a relatively small degree of hydrolysis (form IIa, 24.6-30.8%; form IIb 21.6-24.8%), there was no evidence for the presence of high molecular weight α-D-glucans (eluting in the vicinity of the void volume) in the enzyme-resistant fractions of these materials. It would appear, therefore, that enzyme action on amylose-lipid complexes proceeds more uniformly than with granular starch, so that no intact particles of the complexes exist at any time. Although α-amylolysis is expected to involve the same molecular mechanism of catalysis, BSA seems to have greater access to the amorphous regions of the complex superstructure than to the lattice of granular starch. Between the two enzymes, the BSA gave slightly broader chain distributions and of lower molecular weight for the enzyme resistant fragments than HPA. Moreover, the peak fractions of the eluting chains (Figure 32) for all samples were greater than DP 45, in agreement with previous reports (Biliaderis and Galloway 1989; Galloway *et al.* 1989; Jane and Robyt 1984) on structure characterization of such residues.

Physical properties of enzyme-resistant fractions

To further elucidate the mode of chain degradation of complexes by α-amylases in the solid state, X-ray diffraction measurements and thermal analysis by DSC on the α-amylolysis residues were performed. In Figure 33 the wide angle X-ray diffraction patterns of native forms I, IIa and IIb as well as their enzyme treated counterparts are presented. There have

Figure 33. X-ray diffraction patterns of wet glycerol monostearate-amylose complexes: (a) Form I, (b) Form IIa, and (c) Form IIb; numerical subscripts correspond to native (1) and residues of *B. subtilis* (2) and hog pancreas (3) α-amylase-treated complexes (48h, 3.3IU/100mg complex). Percent hydrolysis of V-amylose for the enzyme-treated residues were: (a_2) 70.8; (a_3) 80.2; (b_2) 24.6; (b_3) 30.8; (c_2) 21.6; (c_3) 24.8.


Intensity
c3
c2
c1
b3
b2
b1
a3
a2
a1
6
16
26
Diffraction Angle (2θ°)

been no substantial changes in crystallinity of the residues after enzymic treatments at a specified level of hydrolysis (Figure 33), except for some broadening of the diffraction peak at 13.1 $2\theta^0$ for the IIa and IIb forms. The DSC data of wheat starch and complexes for BSA and HPA treatments are summarized in Tables 6 and 7, respectively. While DSC analysis on the residues of the BSA digests was carried out directly, cold extraction of the released monoglycerides (upon hydrolysis) with $CHCl_3$ was chosen in the case of HPA residues to overcome noisy DSC signals. There was a progressive reduction in the melting enthalpy of all forms of the complex as hydrolysis with BSA proceeded. This was accompanied by a concomitant increase in the melting transition of liberated (uncomplexed) monoglyceride (Table 6). The presence of free lipids (following α-amylase hydrolysis of the complex) in the residues is not surprising when considering the high affinity of starch for adsorbing aliphatic molecules, as was demonstrated by electron spin resonance spectroscopy (Biliaderis and Vaughan 1987). A slight reduction in the transition temperatures was also observed with increasing hydrolysis time. For wheat starch granules, on the other hand, although initially showed smaller enthalpies of gelatinization, after 72hr of digestion the recovered residues had comparable transition enthalpies to those of the native sample. Colonna *et al.* (1988) also reported similar DSC thermal profiles for native and BSA treated wheat starch granules. The DSC data of Table 6, therefore, confirm the notion for a different mode of degradation of these materials (complexes vs. wheat starch granules) by the *B. subtilis* α-amylase discussed above. Interestingly, the enthalpies of amylose-lipid (internal starch lipids) transitions of wheat starch showed a progressive reduction

TABLE 6. DSC characteristics (20 w/w in H_2O solids) of wheat starch granules and glycerol monostearate-amylose complexes after hydrolysis with *Bacillus subtilis* α-amylase (0.5g/60ml 0.1M buffer, pH 5.5, 37°C, 3.3IU/100mg complex)

Sample	Parameter	Time (hr)					
		0	5	14	24	48	72
Wheat starch	$\Delta H_{gelatin}$	7.5±0.1[a]	5.8±0.2	5.0±0.7	6.2±0.6	6.7±0.8	7.0±0.3
	$Tm_{gelatin}$	60.1±0.2	61.1±0.3	64.6±0.3	62.7±0.1	65.5±0.8	64.3±0.2
	$\Delta H_{complex}$	4.6±1.2	4.0±1.3	2.4±2.0	2.5±0.2	1.9±0.6	1.4±0.4
	$Tm_{complex}$	98.6±0.3	97.6±0.2	98.4±0.1	97.±90.5	94.8±1.9	93.4±0.9
Form I	ΔH_{lipid}	0.6±0.1	2.7±0.6	4.6±0.5	9.0±0.2	18.2±0.2	25.9±2.2
	$\Delta H_{complex}$	20.5±0.2	19.3±0.5	14.5±0.2	12.8±0.1	8.3±1.5	4.1±0.3
	$Tm_{complex}$	98.9±0.1	97.7±0.3	97.2±0.1	96.9±0.3	94.8±0.4	93.9±0.5
Form IIa	ΔH_{lipid}	0.2±0.1	0.5±0.2	3.4±0.6	2.9±0.6	2.7±0.3	5.2±1.1
	$\Delta H_{complex}$	23.7±1.6	23.6±0.9	22.4±1.1	20.8±1.9	19.7±0.9	18.1±0.9
	$Tm_{complex}$	114.4±0.3	112.9±0.3	111.7±0.5	111.3±0.6	110.7±0.7	110.5±0.3
Form IIb	ΔH_{lipid}	0.2±0.1	0.2±0.2	0.9±0.6	1.6±0.3	1.6±0.2	1.3±0.2
	$\Delta H_{complex}$	26.7±0.3	26.5±1.2	24.0±0.6	24.0±1.6	23.7±0.1	22.1±1.3
	$Tm_{complex}$	124.6±0.6	124.3±0.3	123.2±0.2	122.6±0.1	122.6±0.5	122.3±0.4

[a] Means ± s.d (n=3)

TABLE 7. DSC characteristics (20% solids w/w in H_2O) of wheat starch granules and glycerol monostearate-amylose complex after hydrolysis with hog pancreas α-amylase (0.5g/60ml buffer, pH 6.9, 37°C, 3.3 IU/100mg complex) and removal of liberated monoglyceride by cold $CHCl_3$ extraction.

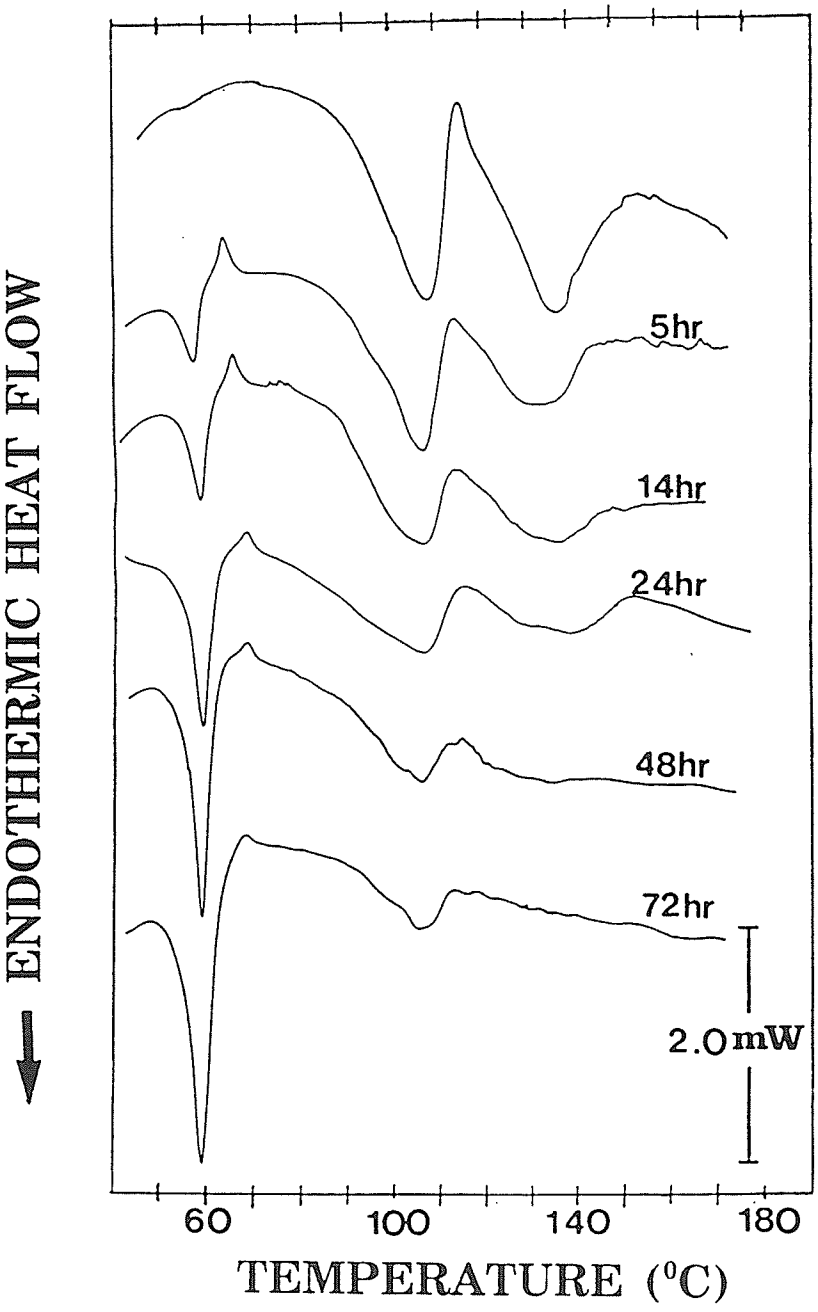
Sample	Parameter	Time (hr)					
		0	5	14	24	48	72
Wheat starch	$\Delta H_{gelatin}$	7.5±0.1[a]	6.4±0.4	6.5±0.9	4.6±0.3	4.2±1.3	3.0±0.4
	$Tm_{gelatin}$	60.1±0.2	62.3±0.3	65.7±0.1	63.6±0.3	65.1±0.3	66.9±0.6
Form I	$\Delta H_{complex}$	20.5±0.2	19.1±0.5	16.5±0.2	10.0±2.1	4.0±0.8	-
	$Tm_{complex}$	98.9±0.1	98.8±0.5	96.4±0.9	95.2±0.4	93.2±0.5	-
Form IIa	$\Delta H_{complex}$	23.7±1.6	23.3±0.3	22.6±0.5	20.7±0.2	16.1±1.1	11.7±0.6
	$Tm_{complex}$	114.4±0.3	112.7±0.1	110.4±0.3	109.4±0.1	108.9±0.2	108.1±0.1
Form IIb	$\Delta H_{complex}$	26.7±0.6	26.5±0.7	24.1±0.4	22.4±0.6	21.8±0.7	20.6±0.4
	$Tm_{complex}$	124.6±0.6	124.6±0.7	122.2±0.5	121.3±0.2	120.6±0.2	120.0±0.1

[a] Means ± s.d (n=3)

(Table 6). This finding suggests that amylose, being present mostly in the disordered regions of the granules, is more sensitive to enzymic depolymerization than amylopectin crystallites, thus showing diminishing ability for complexation with native granular lipids during heating.

Even more pronounced reduction in the magnitude of melting transitions of complexes was found in the case of HPA residues (Table 7), albeit the X-ray diffractograms showed no changes in crystallinity of these materials (Figure 33). The combined DSC and X-ray data of the present study, therefore, suggest that upon removal of interconnecting amorphous chain segments between crystalline domains of the complexes, the latter are becoming thermally unstable (i.e. exhibit lower ΔH and Tm) compared to their native structures. The importance of interspersed disordered chains to the thermal stability of the complexes is revealed in Figure 34 for form I. It is well known that complexes of amylose with monoacyl lipid exist as metastable states of varying degree of perfection in their ordered domains, depending on the conditions employed during complexation (solvent, temperature, ligand type) (Biliaderis and Galloway 1989). Form I, for example, because of the relatively high degree of supercooling required for its formation (Biliaderis and Galloway 1989), has a supermolecular structure that is amenable to reorganization during heating in the DSC (at 30-60% moisture content and under moderate heating rates) (Biliaderis *et al.* 1985). Such thermal behaviour is manifested by multiple melting endotherms separated by an exothermic effect in the DSC curve, as illustrated in Figure 34 (top curve)for the control sample. After partial melting of the structure (first endotherm), the complex reorganizes into a more perfect form of greater thermal stability (second

Figure 34. DSC thermal curves of solid residues of Form I (50% w/w in H_2O) following hydrolysis with *B. subtilis* α-amylase for a specific reaction time. Mass of complex from top to bottom: 3.95(control), 3.41, 3.91, 4.25, 3.95, 3.61; heating rate 10^0C/min.


ENDOTHERMIC HEAT FLOW
5hr
14hr
24hr
48hr
72hr
2.0 mW
60
100
140
180
TEMPERATURE (°C)

endotherm). It is apparent from Figure 34 that as α-amylolysis proceeds and the proportion of interspersed amorphous chains decreases, there is less tendency for the remaining structure of the complex to reorganize during the DSC heating experiment. Furthermore, there is a progressive reduction in the magnitude of melting transitions of the complex. The sharp endothermic transitions between 55 and 60^0C correspond to melting of the lipids released when the complexes are degraded.

For wheat starch, in contrast to BSA digests, the HPA-treated residues showed a continuous decrease in the gelatinization enthalpies, implying a different mode of action for this enzyme (Table 7). This observation suggests that different hydrolysis patterns may exist among amylolytic enzymes and warrants further investigation on degradation of granular starches using amylases from various sources and examination of enzyme resistant fractions by employing a range of analytical techniques (DSC, X-ray diffraction, electron microscopy etc.).

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SUMMARY AND CONCLUSIONS

The present study was undertaken to investigate the stability, interconversion and enzyme digestibility of glycerol monostearate-amylose complexes and obtain further evidence on the supermolecular organization of these materials.

As with synthetic polymers, whose mechanical behaviour is determined by a number of structural parameters (molecular weight, branching, cross-linking, crystallinity and crystalline morphology, molecular orientation, and plasticization, Mandelkern 1984), the thermomechanical properties of amylose-lipid complexes in bulk or in a composite starch containing product would be dependent on their supermolecular organization, i.e. the way chains intermingle to give ordered and disordered domains in the bulk solid state. Such higher order of chain organization is extremely complex and rather difficult to describe morphologically, since the polymer chains can exist in various aggregated states, depending on the crystallization conditions, molecular weight distribution, and polymer polydispersity. Different structural forms of amylose-lipid complexes are now being recognized and need to be studied in a systematic manner if one is to establish relationships between properties and morphological features of such non-equilibrium states. The DSC, X-ray and structural analysis data presented in this thesis further supported the proposal of earlier work that amylose-lipid complexes, in the solid state, exist mainly as two morphologically distinct supermolecular, metastable structures (Biliaderis

and Galloway 1989). Form I, a kinetically preferred polymorph, since its Tm does not vary, seems to consist of helical-chain segments of very little crystallographic register with one another. A diffused two-line V-diffraction pattern obtained from this form upon lyophilization suggested the formation of a more ordered tertiary structure via chain aggregation during freezing. However, such limited inter-chain associations in the solid state were insufficient to cause changes in the thermal properties of Form I. Instead, Form I seems to be a separate thermodynamic state (between those of liquid/melt and classical crystals) that converts into form II only after partial melting of its structure; i.e. large energy barriers exist between form I and II, which confer kinetic stability into the system. Form II, on the other hand, exhibits a well defined three-line V-diffraction pattern, typical for this particular crystalline structure. Crystallites of this form can be perfected/enlarged via isothermal annealing.

The conformational responses of the two forms in the presence of sulphate and sucrose indicated that the structure of the complex was stabilized as reflected by increases in Tm for both forms. Similar trends were also found for glucose and a series of maltooligosaccharides. At high concentration of these solutes (e.g.> 1.17M sucrose) the metastable form I underwent reorganization during thermal analysis (10^0C/min; complex 20% w/w in solution). The exothermic event between the two endotherms is indicative of reorganization of the complex into a state of lower free energy. These results also implied that phase transition of these materials are far from equilibrium or even zero-entropy production melting. In contrast to Na_2SO_4 and sugars, the DSC thermal curves of form

I and II in the presence of urea and guanidine hydrochloride exhibited a progressive destabilization of the structure with increasing concentration of the dissociating agent; i.e. the Tm and ΔH decreased progressively and the transitions became broader.

The relative ranking of the anions (at concentrations < 1.0M) in promoting conformational disordering of the two structural forms was: SCN^- > I^- > NO_3^- > F^- > Cl^- > CH_3COO^- > SO_4^{--}. This order is in accord with the lyotropic or Hofmeister series. The thermal responses of form I and II, to the series of anions examined exhibited similar trends at low salt concentrations. However, the responses to CH_3COO^- varied between the two forms; while this ion stabilized form I over the entire concentration range, it lowered the Tm of form II at high salt concentrations (>2.0M). This implies that CH_3COO^- acts differently at various levels of superstructure of the complex. Cations, on the other hand exerted stabilizing effects on both complexes; the ranking of the cations in terms of their ability to stabilize the complex structure was $NH_4^+ < K^+ < Na^+ < Li^+ \leq Ca^{2+} \leq Mg^{2+}$. Among the various cations tested, CsCl exhibited mild dissociating effects with respect to the superstructure of the complex. While this salt stabilized form I (0.2-7.0M), it caused disruption of the aggregated helices of form II between 4.0-5.0M. At much higher concentrations, single endotherms were seen, which corresponded to the melting of form I. These results suggest that CsCl promotes disaggregation of amylose-lipid helices while maintaining the conformational order in individual helices. In dealing with transition dynamics among the various metastable forms of the V-complexes, the stability and interconversions in the aggregated state would depend on how the various molecular and

environmental factors affect the aggregation/dissociation of helices as well as the helix-coil transitions.

The possibility that differences in structure between forms I and II might influence the susceptibility of V-amylose to α-amylases was examined by employing α-amylases from *B. subtilis* and hog pancreas. Form I was preferentially degraded in comparison with complexes of greater degree of crystallinity (i.e. Forms IIa and IIb). However, even the crystalline forms of the complexes were fully degraded under prolonged digestion times and high enzyme levels. Chromatography of the resistant amylodextrins also revealed distributions of longer chains for the more perfected complex superstructures. The DSC and chromatography data on enzyme resistant fractions indicated that the *B. subtilis* α-amylase exerts a more uniform hydrolytic action on complexes, compared to wheat starch granules. These findings along with X-ray diffraction and DSC data indicated that the degree of organization of helical chain segments (supermolecular structure) affects the susceptibility of amylose-lipid complexes in the solid state to α-amylolysis.

CONTRIBUTIONS TO KNOWLEDGE

1. Two distinct supermolecular metastable structures of glycerol monostearate-amylose complexes (form I and II) were identified and characterised by thermal analysis, X-ray diffraction and other analytical techniques. The results of this study indicated that these two forms represent two separate thermodynamic states for the complex superstructure.
2. Conversion of form I to II can be carried out by isothermal annealing at temperatures above the melting point of form I .
3. The enthalpy changes during thermal dissociation of the complex represents mainly the energy required for helix→coil transitions.
4. The conformational responses of the complex in various stabilizing and destabilizing environments suggested that stability and interconversions between the various forms can be explained by considering that structural order at two levels (intramolecular and supermolecular) is affected.
5. The effectiveness of anions and cations in stabilizing or destabilizing the ordered chain domains of the complex follows in general the classical Hofmeister series. Certain ions, however, differ in their action between the two forms of the complex.
6. Glucose and malto-oligosaccharides were effective stabilizers of the complex and resulted in non-equilibrium thermal dissociation of all supermolecular structures. These effects were proportional to the molecular weight and concentration of the carbohydrate solute.

7. The rate and extent of hydrolysis of the complex by α-amylases in heterogeneous reaction mixtures were inversely related to the degree of organization of helices into ordered chain domains in the solid state.
8. Enzymic breakdown of amorphous chain segments of the complex resulted in reduced thermal stability (lower enthalpy and dissociation temperature) of the enzyme-resistant residues without any substantial change in their crystallinity.

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