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APPLICATION OF A MODIFIED I.G.C. METHOD IN THE STUDY OF THE WATER SORPTIONAL BEHAVIOR OF SELECTED PROTEINS I. LYSOZYME-WATER INTERACTIONS.

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SUMMARY

The water sorptional bahavior of a hydrophilic protein (purified lysozyme) as affected by sample pretreatment (slow freezing, fast freezing, glycosylation and predrying) was studied using a modified pulse inverse gas chromatographic method. Fast-frozen lysozyme showed a slightly higher water adsorption than slow-frozen one, where glycosylated lysozyme adsorbed less compared to the unglycosylated sample. On the other hand, predrying of the substrates caused a relatively significant diminishing on their sorptional behavior because of structural changes (restructuring) occuring during predrying. Data were succesfully fitted to the Guggenheim, Anderson and De Boer (G.A.B.) equation and analysed according to the Zimm-Lundberg cluster theory. Results showed enhanced cluster formation tendency for predried materials as well as for glycosylated lysozyme.

INTRODUCTION

In recent years, protein-water interactions have received increasing attention as these interactions play an important role in determining the three dimentional structure of the protein molecules as well as many of the functional properties of proteins in foods (refs 1-3). Water molecules are linked to the active site of the substrate changing the individual structures to more cohesive and thus affecting the physical properties of fabricated foodstuffs. The amount of water absorbed depends on the type of protein, the degree of bioactivity (native or denaturated) and the conformational (spacial) structure of protein (ref. 4).

Lysozyme is a well-known protein with approximate molecular weight of 14,400. Each molecule consists of a single polypeptide chain with 129 aminoacids, the main of them being arginine (8.5%), aspartic acid (16%), threonine (8%), glycine and alanine (ref. 5). The protein exhibits high hydrophilicity and is stable to heat up to 55°C. Thus lysozyme serves as a good substrate for investigating protein-water interactions involving polar binding sites (refs 6-8)).

Numerous methods have been used to study water binding by proteins or, generally, the properties of protein-water interaction. The simplest and most accepted technique used to study these properties is sorption-desorption isotherm, e.g. graph of water activity (a_w) versus moisture content at constant temperature. Water sorption isotherms of protein systems can give the necessary knowledge to understand the mechanism of water sorption and the role of water in some reactions critical for the performance of commercial foods such as Maillard reactiosn etc (refs 4,9,10).

Water sorption isotherms can be analysed with theoretical models derived from physicochemical calculations. Specifically, this analysis can provide values for monolayer coverage of the substrate, specific active sites and clustering levels at variable coverage.

The use of the method of inverse gas chromatography (iGC) for the determination of sorption isotherms is especially advantageous when there is a need for rapid water sorption determination and limited amounts of substrates are available (ref. 11). The rapidity of the method has been further inhanced by using a recently developed modified pulse technique which produces a sorption isotherm from a single chromatographic elution peak (ref.12). Moreover, the method does not require that moisture equilibrium be attained during data collection. Based on mass balances and equations well described elsewhere (ref. 9,12,13) the evaluation of water uptake, water content and water activity are based on the following mathematical relations:

$$W_u = \frac{k_t\, t_i - k_a\, A_i}{m_s} \qquad (1)$$

where W_u is the water uptake (g/g); m_s is the amount of stationary phase (g); t_i is the time (s); Ai is the area under the elution peak at time ti; kt is time constant; and ka is area constant,

$$a_w = \frac{V_{ti}}{V_{max}} \qquad (2)$$

where V_{ti} is the voltage at time ti; and V_{max} is the maximum peak voltage in an empty (calibration) column under the same conditions.

The objectives of the present study were:

a) To use the new modified IGC technique for determination of water sorption isotherms of various lysozyme samples.

(b) To study the effect of pretreatment (fast freezing, slow freezing and predrying) on the water sorptional behaviour of purified lysozyme.

(c) To gain new insight on posible water-water and water-protein interactions by using model equations (G.A.B) and Zimm-Lundberg cluster analysis.

MATERIALS AND METHODS

MATERIALS

Purified slow-frozen and fast-frozen samples were prepared as follows: A preweighed amount of lysozyme (Sigma Chem. Co., cat. #6876) was dissolved in deionized water and filtered through 0.45 micron cellulose acetate filter (Millipore, type HV). The sample was dialyzed against deionized water using a preconditioned dialysis tubing (boiled six times in deionized water using a preconditioned dialysis tybing (boiled six times in deionized water) of molecular weight cut-off (MWCO) 3,500, for 4 days at 4°C, changing water 1-2 times per day. A portion of the sample was slow-freezed and freeze-dried for 48 hours at lowest vacuum. (slow-frozen lysozyme). Another portion of the sample was immersed in a dry-ice bath for a few seconds (fast-frozen lysozyme).

Glycosylated slow-frozen lysozyme was prepared as follows: 1g purified slow-frozen lysozyme and 3000mg anhydrous D-glucose (Analytical grade) were dissolved in 15 ml of deionized water, slow-freezed (overnight in freezer) and freeze-dried for 48 hours at lowest vacuum. Lyophilized material was placed into chamber under controlled atmosphere equilibrated at 65% R.H. and 50°C for 15 days. After this, 15 ml of water were added to the reaction vial and the sample was dialyzed using dialysis tubing of (a) MWCO = 1,000 for 8 hours, (b) MWCO = 3,500 for 8 hours and (c) MWCO = 10,000 for 8 hours. Aliquots were collected each two hours and the disappearance of glucose was measured using a spectrophotometric test (Methods in Enzymology, 1984, Vol.116, 77-87). The sample was finaly slow freezed and freeze-dried for 48 yours at lowest vacuum.

The predried materials for each of the above samples were prepared by packing an appropriate amount of them into a GC column which was placed into the GC oven at 55°C and subjected to 1 hour drying under inert gas flow adjusted at 40ml N_2/min to a presumed constant weight since water vapor pressure of sample was below the sensitivity of the thermal conductivity detector.

METHODS

1. IGC instrumentation and procedure

A schematic diagram of the setup equipment used in the present study is shown in Figure 1. The gas chromatogram was a Varian 3700 equipped with a thermal conductivity detector and a liquid carbon dioxide cryogenic system. The analog to digital (A/D) converter,

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model Dash-8 (12 bit resulution) and the analog input submultiplexer expansion, model EXP-16, were purchased from Metrabyte Co (Tannton, MA, USA). The personal computer was an IBM-XT (International Business Machines Corp.). The Labtech Notebook software (Laboratory Technologies Corp., Cambridge, MA) version 4.1, was used for the data acquisition, while for the data analysis the Lotus 1-2-3- program, version 2.01 was used.

The GC columns were pure-sample and short lenght. A swagelok reducer fitting was used as column loaded with approximately 50 mg of sample with the aid of a vacuum pump and a mechanical vibrator. A relatively large amount of solute e.g. 25-30 μl water) was injected using an empty (calibration) column first and then using a loaded one. The analog response from the GC was digitized while displayed in real-time mode on the monitor screen and simultaneously was stored in a real ASCII file on the computer's hard disk. Further analysis of data to obtain water sorption isotherms was performed using a BASIC language program.

Model equation analysis was carried out using the Labtech's Notebook curve-fit (non-linear regression analysis) program.

2. Density measurements

True density measurements (for cluster analysis) were carried out using a gas pycnometer (Quantachrome, model SPY-2 stereopycnometer) which utilized helium gas (20 psig) to measure the true volume of a preweighed aliquot of the sample).

RESULTS AND DISCUSSION

1. Water Sorption isotherms

Figures 2-4 show the GC profiles of "as is" and "predried" materials studied (fast-frozen lysozyme, slow-frozen lysozyme and glycosylated lysozyme at 25°C. The corresponding water sorption isotherms for the above materials at 25°C are given in fugures 5-7. Figures for fast-frozen lysozyme samples show the high sorption capacity of highly amorphous lysozyme as well as the transition occuring at abour 65% R.H. when water organizes in clusters. This restructuring effect is more pronounced in case of "as is" sample and depends on the extent of exposure time to permit water cluster formation into the matrix of the substrate and also on temperature. In case of slow-frozen lysozyme (Figs 2 and 5), the observed transitions were much less pronounced. This can be attributed to the significantly less matestable structure obtained by the slow-freeze procedure. Similarly, transitions have not been observed in case of glycosylated lysozyme (Figs 3 and 6) indicating more stable structure. Above results are in general accordance with previous experimental results on similar substrates (refs 9,13) and

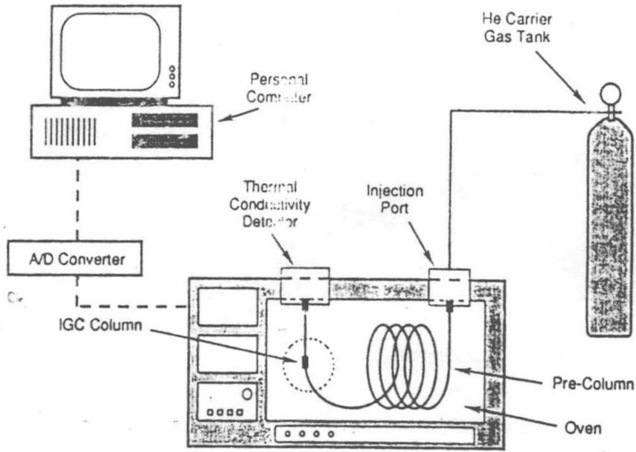

Figure 1: Schematic diagram of the I.G.C. setup equipment

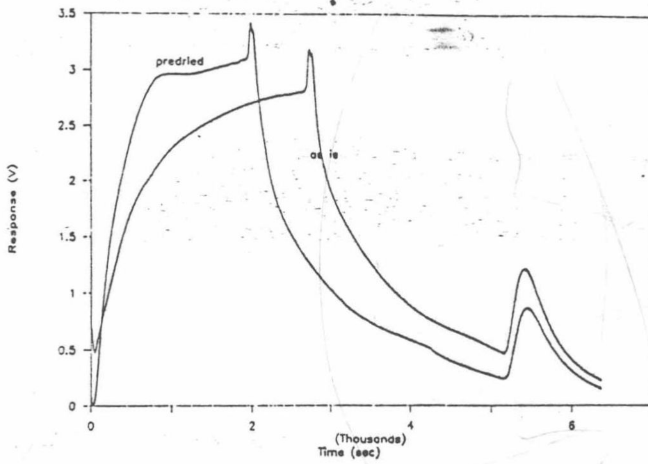

Figure 2: Chromatograms of "as is" and "predried" fast-frozen purified lysozyme samples at 25°C.

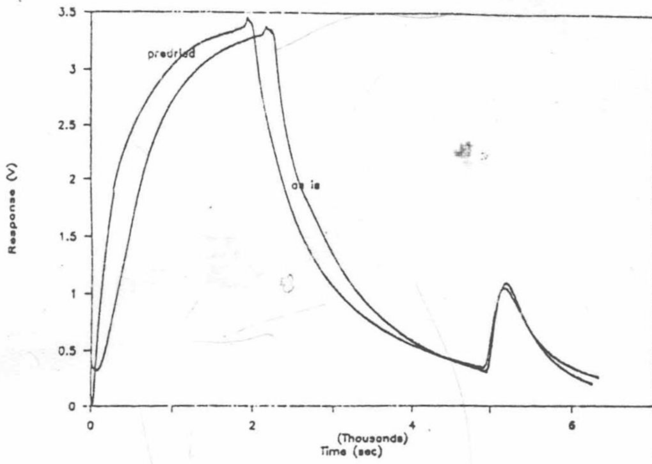

Figure 3: Chromatograms of "as is" and "predried" slow-frozen purified lysozyme samples at 25°C.

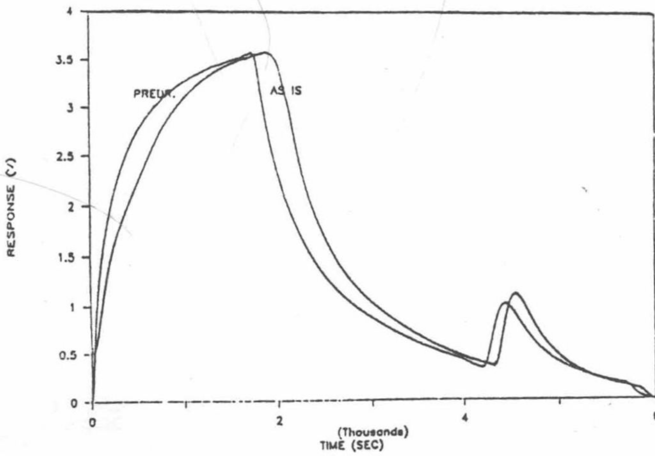

Figure 4: Chromatograms of "as is" and "predried" glycosylated slow-frozen lysozyme samples at 25°C.

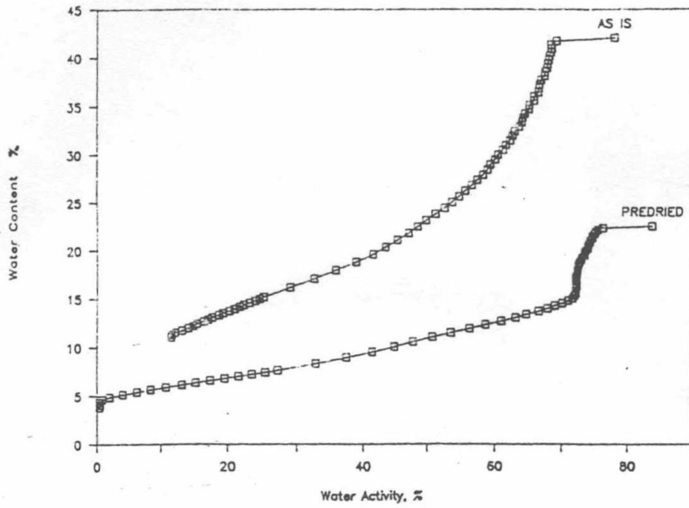

Figure 5: Water sorption isothrems of "as is" and "predried" fast-frozen purified lysozyme samples at 25°C.

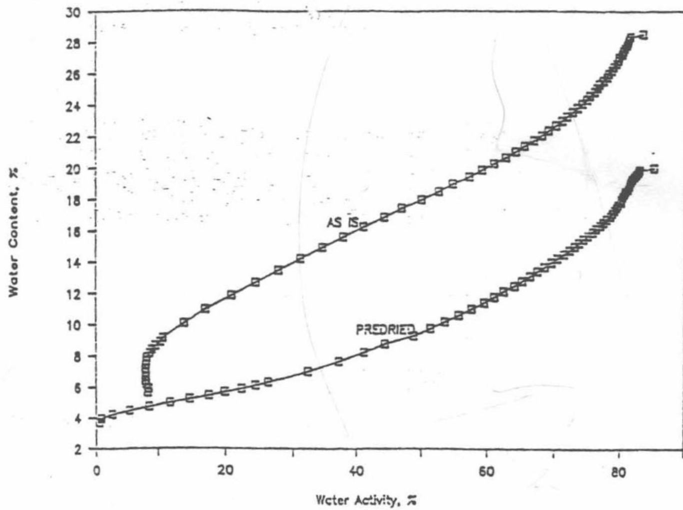

Figure 6: Water sorption isothrems of "as is" and "predried" slow-frozen purified lysozyme samples at 25°C.

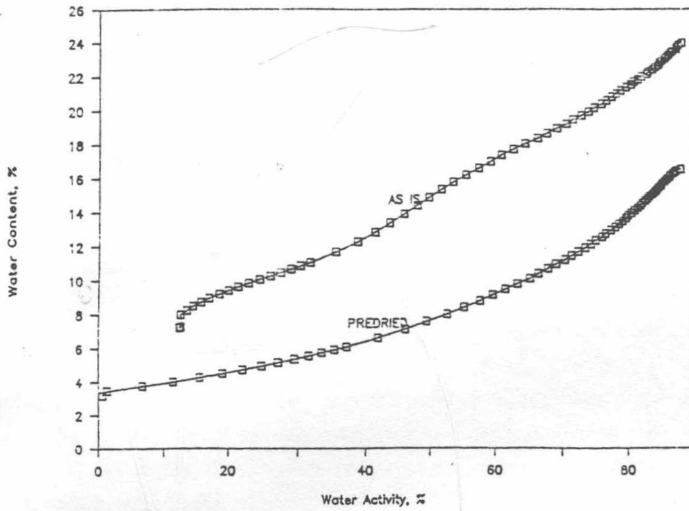

Figure 7: Water sorption isothrems of "as is" and "predried" glycosylated slow-frozen lysozyme samples at 25oC.

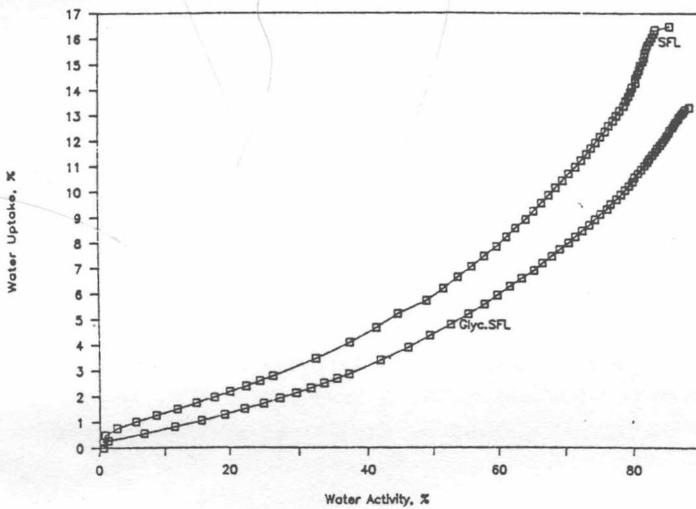

Figure 8: Comparison between water sorption isotherms of "predried" slow-frozen lysozyme and glycosylated lysozyme samples at 25oC.

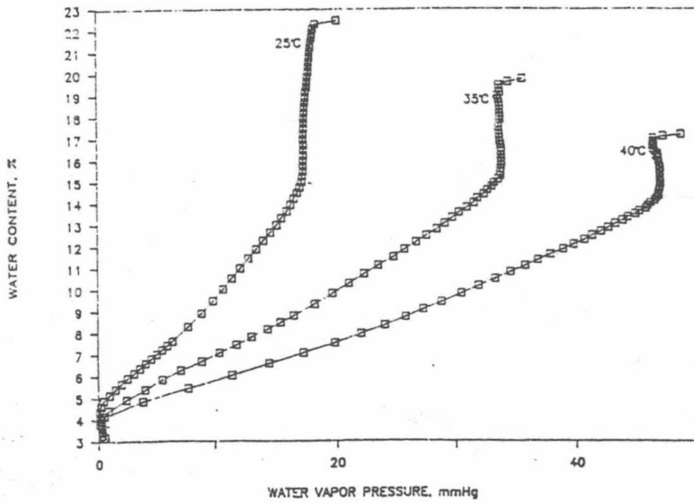

Figure 9: Water sorption isotherms of fast-frozen purified lysozyme at different temperatures.

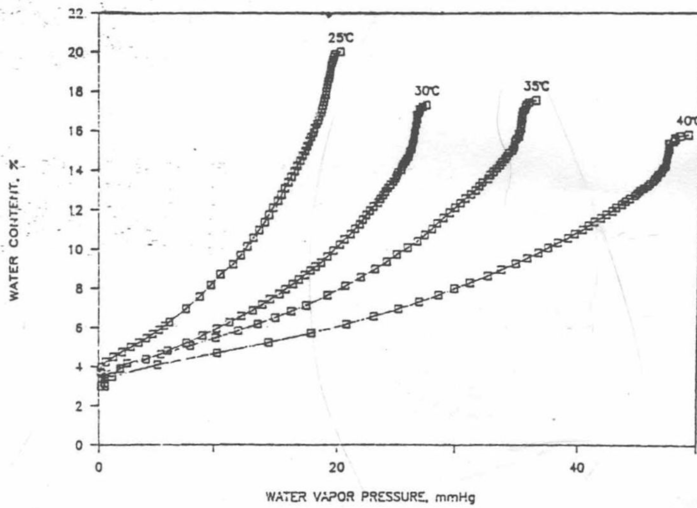

Figure 10: Water sorption isotherms of slow-frozen purified lysozyme at different temperatures.

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