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X-Ray Crystallography and Its Applications in Dairy Science: A Review

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Abstract

X-ray crystallography (XRC) is the study of crystals using X-rays. XRC is the primary method in which detailed structure of molecules especially molecules that pertain to living systems have been visualized and discovered by exposing a well-ordered crystal of a substance to X-rays and finally generating the structural information from the spots produced on a film due to this impact. X-ray crystallography has been used for analysis of liquid milk, milk powders, milkstones, polymorphism of milk fat and most widely and importantly in discovering the structure of most of the milk proteins and thus helping in correlating their structure with possible functions.

Keywords: *X-ray crystallography, crystals, detector, resolution, electron density maps*

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INTRODUCTION

X-rays have short enough wavelengths to "see" the atoms and the molecular structure of molecules. Any structure can be visualized only if electromagnetic radiation of a wavelength comparable to its dimensions is used. For proteins, appropriate size is of the order of Å (10^{-10} m). Wavelength of X-rays (1 to 100 Å) approximates to this; hence, use of X-ray crystallography technique is useful for studying structure of biological macromolecules.

X-ray crystallography is the most widely used technique to obtain high-resolution protein structural information. It is the study of crystals using X-rays. XRC is the primary method in which detailed structure of molecules especially molecules that pertain to living systems have been visualized and discovered by exposing a well-ordered crystal of a substance to X-rays and finally generating the structural information from the spots produced on a film due to this impact.

Crystals are not a requirement of analysis using X-rays; any ordered (or partially ordered) array of molecules can produce useful X-ray data. But, crystals are the most favourable samples. So, crystals have become the prerequisite for X-ray diffraction experiments. Production of suitable crystals is rate limiting for study of many proteins [1]. High purity of protein preparations used for crystallization is the most important factor for growing diffraction quality crystals.

X-RAY CRYSTALLOGRAPHY/ X-RAY DIFFRACTION

It is a technique which relies on the dual nature (wave/particle) of X-rays to discover information about structure of crystalline materials. In this technique, the pattern produced by the diffraction of the X-rays through the closely spaced lattice of atoms in a crystal is recorded and then analyzed to reveal the nature of that lattice. This technique entails bombarding a sample of protein in crystalline form with a beam of X-rays. Most of these X-rays pass straight through the crystal but some are diffracted by it, resulting in a diffraction pattern recorded on a detector (Figure 1). This diffraction pattern is a reflection of three-dimensional structure of protein molecule present in the crystal. X-ray crystallography can provide very detailed atomic information, showing every atom in a protein or nucleic acid along with atomic details of ligands, inhibitors, ions, and other molecules that are incorporated into the crystal.

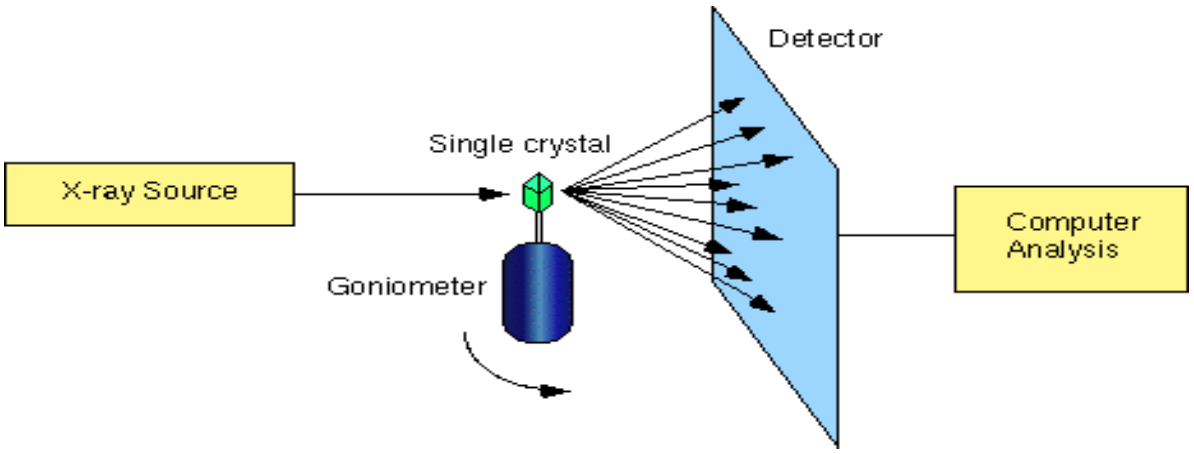


Fig 1: *Schematic of X-Ray Diffractometer.*

Basic Method of X-ray Crystallography

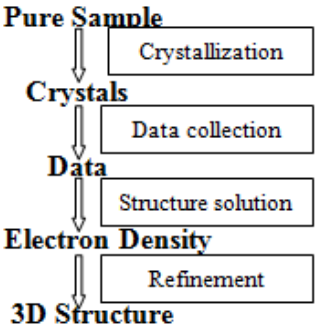


Fig 2: *X-Ray Crystallography Method to Obtain 3D Structure of Molecules.*

Crystallize the molecule, subject it to irradiation by a beam of X-rays and make a record of the 3D diffraction pattern. Analysis of this data through computer calculations results in a model of molecule which can then be refined against observed X-ray data. Finally, one obtains 3D coordinates which describe position in space of each atom in the molecule (Figure 2). Such a refined model can be displayed and manipulated either as plastic or wooden models or on a computer graphics format.

Generation of X-Rays

X-rays can be generated when a metal plate is bombarded with accelerating electrons. This is normally achieved in high voltage tubes, but more powerful X-ray beams may be generated in synchrotron storage rings, using electrons traveling with the speed of light.

X-ray source is usually a sealed tube in which electrons are accelerated from one end and allowed to impinge at other end on a metal target, usually copper or molybdenum for biologically relevant samples. This produces X-rays of wavelength 1.5418 Å (for Cu) and 0.7107 Å (for Mo) (Figure 3).

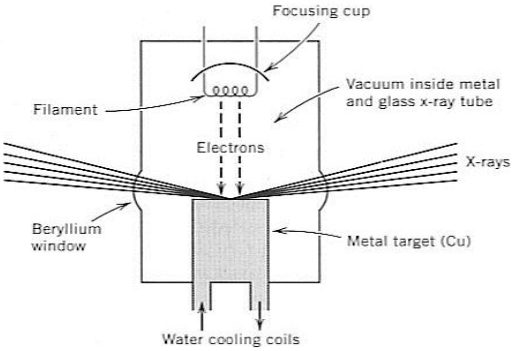


Fig. 3: *X-Ray Generation Using X-Ray Vacuum Tubes.*

Diffraction by X-rays is described in terms of reflections from crystal plane. Diffraction occurs when two or more waves are combined.

Bragg's Law

Bragg's law is a fundamental law and is valid for monochromatic X-rays only and is used to calculate interplanar spacing used in XRD spectra. It defines the spacing (d) of atomic planes and incident angle (θ) at which X-rays of a particular wavelength will reflect in phase (i.e., diffract) (Figure 4). For constructive interference to occur, difference in path lengths given by BC + BD should be equal to an integral number of wavelength of the incident beam.

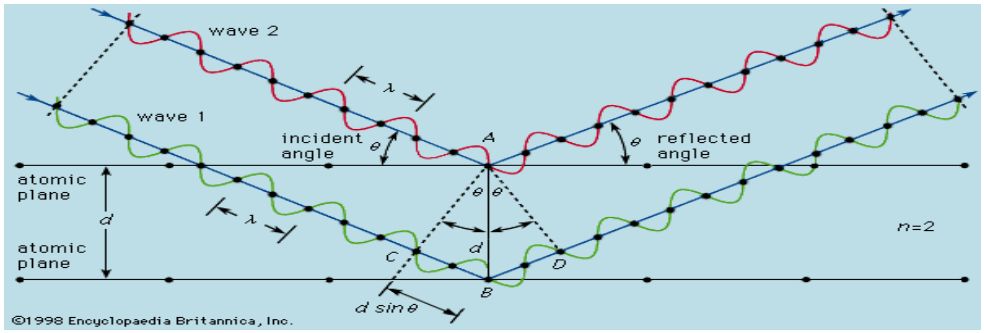


Fig. 4: *Bragg's Reflection Condition or Bragg's Law.*

$BC = BD = d \sin\theta$
$2d \sin\theta = n\lambda$

where, n = an integer
λ = wavelength of the X-rays
d = interplanar spacing in the specimen
θ = diffraction angle
$\sin\theta \propto 1/d$ (reciprocal space)

When a crystal is irradiated by an X-ray beam, Bragg's law is used to predict the angle θ at which diffracted intensities may be expected. This may require the knowledge of inter-planar spacing d and λ of X-rays. Conversely, if angles of occurrence (and relative intensities) of diffracted spots are measured in an experiment, corresponding inter-planar spacing in crystal may be calculated and lattice structure can be determined.

Steps to Determine Structure of Molecules

1. Prepare suitable crystals of native macromolecules. Using crystals, one can determine the space group and then collect a set of scattering amplitude data.
2. Prepare several different heavy atom isomorphous derivatives (having same unit cell, space group and macromolecular structure as parent crystal, except that one or more heavy atoms have been introduced at specific loci). For each derivative, collect a new set of scattering amplitude data.
3. Attempt to find location of heavy atoms in crystals.
4. Refine the positions using Fourier refinement technique.
5. Phases of each F (h, k, l) can be estimated comparing the structure factor data of parent crystal seven heavy-atom isomorphous derivatives.
6. Further refine the position of heavy atom using least square of difference Fourier technique.
7. Using estimated phases and observed amplitudes of each F (h, k, l), one can create an electron density map.
8. A model is built of electron density map. As low resolution data (about 5.5–7 Å) is used, so map does not show well resolved structural details.

➔ Then, step 4 and 8 are repeated at higher resolution (2.5–3 Å) until it is possible to construct a molecular model.

9. Further refine the structure using Fourier or least square technique and can also include information about known energetic of protein conformation**.**
10. Check R-factor of the models.

‡ Experimentally determined 3D structure information is stored in various databases as sets of atomic coordinates.

‡ Principal of such database is Brookhaven protein database (PDB) which may be accessed via internet at http://www.pdb.bnl.gov. There are more than 12,000 sets of crystal structure coordinates for a wide variety of proteins in this data bank.

‡ Various internet sites provide free software programs which can generate protein structural models from atomic coordinates. These structures can be viewed interactively (e.g., rotated and zoomed).

‡ RasMol (http://www.umass.edu/microbio/rasmol) is amongst such best known programs. Another program termed Mage provides interactive protein structural models called "Kinemages" (http://www.fascb.org/protein/kinemage/kinpage.html).

Crystallization

Although many low-molecular-weight substances crystallize relatively easily, this is not the case for extremely larger ones which display irregular surfaces. Even if induced to crystallize, protein crystals will contain solvent-filled channels or pores between individual protein molecules. Only a small proportion of surface of individual proteins interacts with each-other and as a result, crystals are soft and easily destroyed. Production of suitable crystal is rate limiting for study of many proteins.

Growth of Crystals of Biological Molecules

The chief difference between crystals of biological substances and those of other important materials such as silicon is that in the former, repeating units are invariably molecules or groups of molecules. Components required for while in latter, repeating units are atoms or groups of atoms. So, forces of bonding between one repeating unit and another are much weaker in former being Vander Waal's forces or hydrogen bonds. In latter, crystals are built up from strong covalent or ionic bonds. Crystals of biological materials are thus usually soft and brittle than inorganic substances. Another difference is size – biological and organic molecules usually form very small crystals (approximately 1 mm^3) as compared to crystals of inorganic materials (approximately 1 cm^3).

Biological molecule crystals are finicky: some form perfect, well-ordered crystals and others form only poor crystals. Biocrystals are usually grown from solution and may have a large solvent content [2]. This is especially true for crystals of macromolecules such as proteins and nucleic acid, where solvent can take up as much as 80% crystals. Growth of biocrystals is a multi-parameter process.

Factors Affecting Crystallization of Organic Molecules and Biomolecules

‡ Purity of sample
‡ Concentration
‡ Temperature, pH of solution
‡ Time, rate of equilibration
‡ Ionic strength
‡ Convection
‡ Volume of crystallization
‡ Pressure, vibrations
‡ Additives, their character and concentration
‡ Solvents (especially for small molecules)

To grow crystals of any compound from solution, molecules have to be brought to a supersaturated state. When solution returns to equilibrium, substances will precipitate out, hopefully as crystals, not as an amorphous precipitate. By varying one of the parameters listed above, one may change solubility and hence saturation level of molecules. In order to obtain good defect-free crystals of a size suitable for X-ray analysis, equilibration needs to be carefully controlled. A crystal is formed when protein molecules are precipitated very slowly from supersaturated solution and this is usually achieved by slow evaporation, vapor diffusion or dialysis. Final condition of crystallization should be such that the crystallized state is thermodynamically most stable. Crystal growth is not well understood and almost entirely empirical rules are followed to grow crystals.

Methods of Crystallization

Slow Evaporation Technique

Slow evaporation technique is conceptually the simplest technique to grow crystals from supersaturated solutions. It is chiefly used to crystallize small organic molecules. Solution is placed in a small glass beaker and sealed with an airtight cover, except one or two small apertures through which slow evaporation of solvent takes place bringing the solute to supersaturation and hence to crystallization (Figure 7).

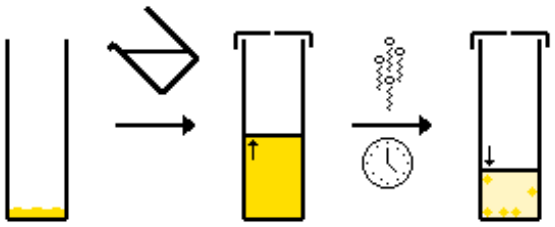


Fig. 5: *Slow Evaporation Method of Crystallization.*

Vapor Diffusion

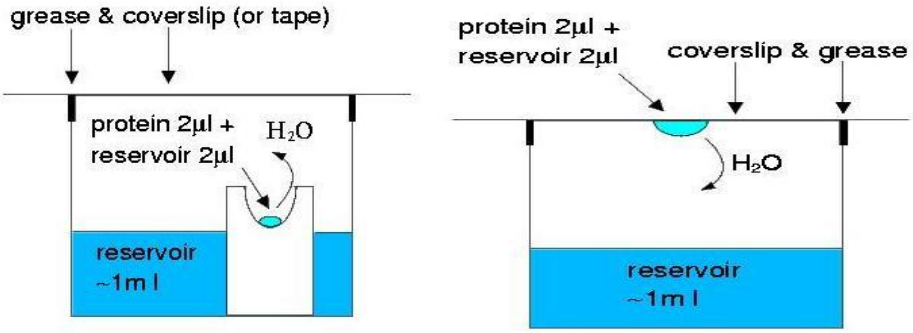
It is the most widely and successful method used for producing diffraction quality crystals.

Vapor diffusion method (Figure 6)

a. Hanging drop method
b. Sitting drop method

A droplet of solution containing the molecule is equilibrated against a reservoir containing the precipitant (i.e., the crystallizing agent at a higher concentration than in the drop) and is slowly dehydrated in a sealed well by equilibration with a reservoir at higher precipitant concentration. In batch methods, the molecule to be crystallized is mixed with crystallizing agent at a high concentration so that supersaturation is immediately reached. Though this may lead to a large number of tiny crystals, it frequently gives rise to good crystals.

A protein drop can either be suspended from the cover slip used to seal the reservoir (a hanging drop) or set on some sort of support above the reservoir (a setting drop). Usually, the drop is made by mixing equal volume of protein solution and reservoir solution.



Hanging Drop Method **Sitting Drop Method**

Fig. 6: *Vapor Diffusion Methods.*

Vapor diffusion method has the benefit of automatically screening a range of precipitation conditions and has a defined end-point. This should allow one to optimize crystallization condition so that the precipitant concentration slowly increases just fast enough to compensate for the loss of protein prior to crystallization. This requires optimization of end-point of experiment and rate of equilibration.

To vary the rate of equilibration is to vary surface to volume ratio (i.e., size) of the drop. In general, larger drops will equilibrate more slowly than smaller drops. However, a general observation is that long equilibration times give better quality crystals.

Interface Diffusion

It is used for small molecules as well as for large biological molecules. Precipitation solution is generally layered over the solution of molecule. Crystals usually grow at interphase. Sometimes, seeds are introduced to induce the crystals to grow. These seeds may be extremely tiny crystallites.

Dialysis

This method has also been quite successful. Precipitant conditions are automatically screened, but unlike previous methods, protein concentration remains constant. Semi-permeable membranes are used to contain protein solution in thick-walled capillary tubes or micro-dialysis buttons. Device is suspended or immersed in a stepwise manner, with an equilibration interval of a few days between steps. This method may lead to crystal growth at a particular height and aid choice of ideal precipitant strength. But, dialysis methods are not economical for proteins (Figure 7).

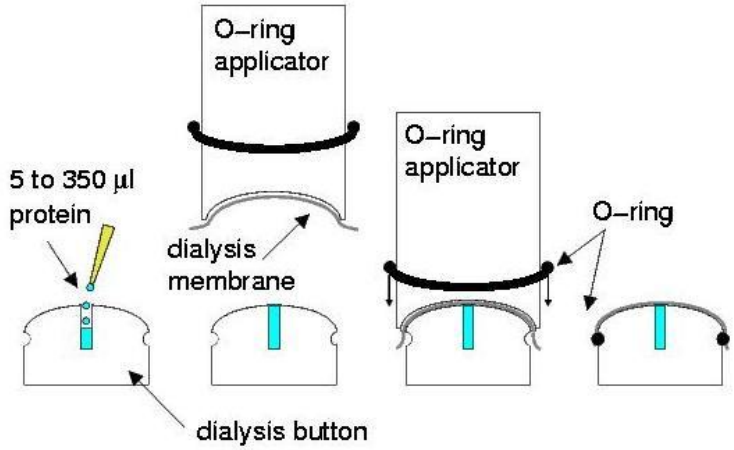


Fig. 7: *Dialysis Experiment.*

Table 2: *Components Required for Crystallization of Biomolecules.*

Molecule	Solvents	Additives	Precipitants
Small organic molecules	Organic solvents (e.g., CCl_4, ethyl acetate, etc.), alcohol, water	Metal ions, salts	Alcohol, organic solvents
Protein	Aqueous solution	Cofactors, sugars, metal ions, complexes, polyamines, salts	Ammonium sulphate, polyethylene glycol (PEG), methyl pentane diol (MPD)
Nucleic acids	Aqueous solution	Polyamines, metal ions, salts, etc.	MPD, PEG, isopropanol and other alcohols.

Micro-heterogeneity in Crystals

Micro-heterogeneity may occur due to presence of some impurities or some local aberrations (dislocations, changes in crystal lattice, and modifications in protein structure) which are visible in the crystal leading to damage in crystal structure and result in relatively weak diffraction patterns. Micro-heterogeneity can have deleterious effects for crystal lattice and its solubility properties and protein might become blurred by such variability, leading to difficulties in defining crystallization conditions.

Detector

Detector of the diffracted X-rays may be a film, or a radiation counter. Films allow recording of several reflections at a time, but suffer from requirement of long exposure times, as well as imprecision in conversion of blackening of film into a numerical value of intensity.

Modern devices such as multi-wire proportional counters or imaging plates offer advantages of both films and counter and are now almost exclusively used in collection of data from crystals of biological macromolecules.

- ➔ Outcome of data collection process is a list of angles, indicating orientations of the crystal and the detector and intensity of reflection from oriented lattice plane at this position.
- ➔ Instead of angles, it is usual to identify the set of planes that give rise to each diffracted spot by Miller indices hkl and associate each value of hkl to the appropriate value of intensity. From the test of intensities, amplitude of each reflected wave is extracted by the simple process of taking the square root of, since the measured intensity of a wave is square of its amplitude. These amplitudes, represented as F_{hkl}, are now used in analysis of the structure of molecules that make up the crystal.

Structure of Molecule

Structure of a molecule is obtained by a Fourier transform of the observed amplitudes F_{hkl}. Fourier transform involves decomposition of a complex periodic function (thick lines) into its sine and cosine components (thin lines). As more component waves at different frequencies (wavelengths) are added, resulting wave approaches a square wave more and more closely. Fourier decomposition of a square wave would result in components of all possible frequencies.

It is not only necessary to know amplitudes (and wavelengths) of simple waves, but also to know the relative positions, or phases of waves in order to reconstruct original periodic function.

To reconstruct the crystal as a 3D superposition of these simple waves of electron density, it is necessary to know the phases. Once phases are known, the mathematical operation of Fourier transform, which corresponds to physical superposition of simple waves, will yield a picture of distribution of electrons within the unit cell of the crystal. In other words, we get a picture of structure of molecules in a unit cell.

In an XRD experiment, phases cannot be measured directly and they have to be determined using indirect methods such as:

‡ Heavy atom or multiple isomorphous replacement (MIR) method

Some direct methods are also available such as:

‡ Molecular replacement method

‡ Anomalous scattering technique

➔ A more recent solution to the phase problem involves using synchrotron radiation at multiple wavelengths. This has greatly accelerated the rate of solving crystal structures.

Refinement of Structure

Once approximate values of phases are determined, next step is to refine them. This is more conveniently done by performing Fourier transform and refining the approximate positions of atoms obtained by including other known data such as stereochemistry. For refinement of the structure obtained by above processes, following methods can be used:

‡ Least square method

‡ Constrained-restrained refinement

‡ Using computer graphics

Resolution of an X-Ray Structure

- Resolution is another model to see how good your model is. Resolution gives the size of the smallest molecule you can see or resolve. It is dependent on the amount of data ultimately phased and used in structure determination. Resolution can be expressed as angular limit at which internal data can be observed by Bragg's law:

$$2d \sin\theta = n\lambda$$

i.e., lower the interplanar spacing d, greater the resolution and higher the angle at which diffracted beam corresponds to that set of planes is observed. Highest resolution achievable using radiation of a particular wavelength is $d = \lambda/2$ Å.

Advantages of XRD Technique

✓ The non-contact, non-destructive nature of X-rays is ideally suited as a probe to characterize materials.

✓ Using diffracted X-rays, knowing the wavelength and diffraction angle, one can make use of Bragg's law, to extract information on the crystalline condition of the sample, and thus phase proportions; texture and degree of preferred orientation can be calculated; crystal structure can be refined using appropriate mathematical models.

✓ Plastic strain and particle size can also be calculated using peak width.

Limitations of XRD Technique besides Phase Problem

⌦ Crystallizing Protein:

‡ Fragile

‡ Requires a crystal with shortest side 0.2 mm

‡ Crystallization may require conditions that cannot be said to be physiological.

⌦ Flaws of Crystallization:

‡ Disorder in Unit Cell

‡ Vibrations of molecules

‡ Distortion in crystallization

- ⌦ Diffraction peaks are very crowded for complex macromolecules, and are difficult to separate.
- ⌦ Heavy atom substitution works for molecules larger than 600 atoms, so a gap is present for molecules in the intermediate size range.

The principal types of information that can be secured by proper interpretation of X-ray data are:

- ‡ Crystalline or non-crystalline substances
- ‡ Crystallographic system, unit cell dimensions
- ‡ Deduction of crystal unit (atom, ions or molecule)
- ‡ Chemical identity, chemical and crystallographic changes
- ‡ Allotropic changes
- ‡ Single crystal or aggregate
- ‡ Type and mechanism of alloy formation
- ‡ Random or fibered aggregate and relative degree of preferred orientation
- ‡ Grain size in an aggregate (in colloidal range)
- ‡ Internal strain or distortion

Basic applications of XRD technique involve:

- ‡ Find structure to determine function of protein
- ‡ Distinguish between different crystal structures with identical compositions
- ‡ Study crystal deformation and stress properties depending on environment conditions
- ‡ Viewing proteins in their native form
- ‡ Seeing difference between primary, secondary and tertiary protein structure
- ‡ Viewing how certain residues would interact and predict subsequent protein folding
- ‡ Study of preferred orientation
- ‡ Study of crystal anisotropy

Molecular Modeling Techniques

Molecular modeling is a group of techniques that employ computer-generated images of chemical structures that show the relative positioning of all the atoms present in the molecule being studied, and/or the simulated dynamics of such molecules together with their ordering through spacetime. Such techniques are of considerable help for understanding many physicochemical properties of molecules, and may also provide clues about their possible role(s), that is, their function, in the organism. They can be thus especially valuable tools for investigating structure-function relationships. X-ray crystallography is an indispensable tool for molecular modeling. Select a target molecule, purify it, and determine structure using suitable technique (e.g., XRD) and computer software program, compare the results with suitable library of structures and get the results. Increasingly, modeling software is available for a variety of industrial applications.

Applications of X-Ray Crystallography in Dairy Science

X-ray crystallography technique has been a widely used tool for elucidation of compounds present in milk and other types of information obtained through structure function relationship. Although more detailed information from X-ray analysis has been secured from substances which are commonly known to be crystalline, it has been surprising to find substances commonly thought of as being non-crystalline as actually having a partially crystalline structure and that this structure can be changed by heat treatment, pressure, stretching, etc. Casein is an example of the latter class of proteins. Stewart [3] has shown that even solutions tend to assume an orderly arrangement of groups within the solution. Hence, liquid milk should, and does show some type of arrangement.The mineral constituent and lactose are the only true crystalline constituents in dairy products that can be analyzed by X-ray; nevertheless, interesting structural changes have been observed in butterfat, milk powder, casein and cheese.

X-ray powder diffraction method is used for powdery substances. Diffraction depends upon the fact that in a fine powder, the particles are arranged in an entirely heterogeneous manner. Since reflection occurs from a definite angle, there should be a sufficient number of particles in the powder turned at just right angle to the primary beam of monochromatic X-rays, to enable strong reflection from one set of parallel planes; other particles turned at another angle will produce reflection from another set of planes (the same set of planes in many particles cooperating). Thus, a beam

passing through a powder specimen will fall upon a perpendicular photographic film as a series of concentric rings, each of same intensity throughout and corresponding to one set of planes of spacing "d".

Analysis of Milk Stones

X-ray diffraction technique has also been applied for analysing the chemical composition of milk stones. Since each chemical compound gives a definite pattern on a photographic film according to atomic arrangement, X-rays can be used for qualitative chemical analysis as well as structural analysis.

X-Ray Analysis of Milk Powder

This technique has also been used in study of milk powder. Most work has been confined to determine the effect of different milk powdering processes upon structural group spacings within the milk proteins. Although, structural changes within the milk protein due to different types of processing equipment are not marked, there is a tendency for shrinkage in unit spacing with an increase in heat treatment. Hence, milk powders made by the roller drying process have a tendency for a smaller d unit spacing than do milk powders of the spray types [4].

Differentiation of Sugar

Since each crystalline compound gives a definite pattern according to the atomic arrangement, the identification and the differentiation of the common sugars (sucrose, dextrose and lactose) is made simple by X-rays [4].

Polymorphism in Milk Fat Shown by X-Ray Diffraction and Infrared Spectroscopy

Occurrence of three polymorphic modifications, viz., α, β' and β was studied by X-ray diffraction and infrared spectroscopy. Excellent agreement between the two methods was obtained. Slow cooling of milk fat resulted in formation of β' and β-forms. Rapid cooling of milk fat resulted in formation of α-form, which upon holding of sample at 5 °C, underwent transformation to β' and β-forms. High-melting fraction (HMF) of milk fat obtained by crystallization from acetone existed in β-form. Slow cooling of melted HMF produced β'-form, and rapid cooling produced α-form. Resolution was improved by removal of liquid portion of fat by pressure filtration. In XRD pattern, a single strong band at 4.05 Å indicates α-form; two strong bands at 4.2 Å and 3.8 Å indicate β-form. HMF of milk fat was obtained by crystallization of a 10% solution in acetone at 15 °C. [5]

Structure Elucidation of Milk Proteins

Milk proteins play a range of roles covering their wide range of nutritional and functional properties. All of these behaviors relate to their structure and possible changes in structure of the component milk proteins during processing. An understanding of the structure of the milk proteins, and how those structures can change under processing conditions, is therefore an important enabling tool for the dairy processing industry.

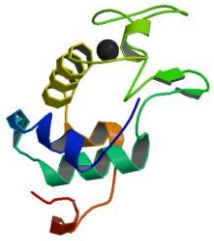
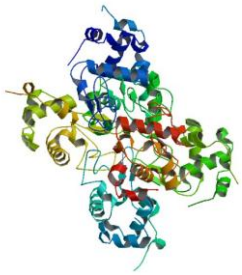
Caseins

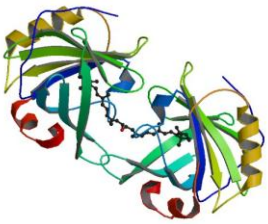
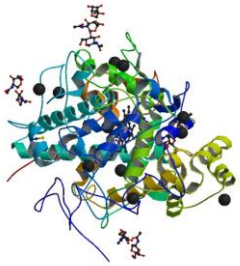
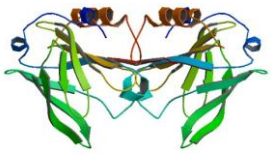
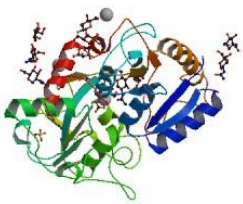
The single feature of casein structure that marks it out as different from other milk proteins is its open and flexible environment-dependent conformation, which has been termed rheomorphic. C-terminus of protein comprises many stretches of consecutive residues with ϕ-χ angles in the region of polyproline II helix [6]. Two such helices could, in principle, combine by hydrogen bonding along the backbone chain, to form a β-sheet with a left handed twist [7], but this does not occur extensively in caseins. This rheomorphic conformation gives the proteins good foaming, emulsifying and gel-forming properties and their remarkable stability to heat. Caseins structure reveals that they do not appear to fall under normal category of globular proteins. To solve the structure of non-globular casein molecules, one way is to form a complex with some other suitable molecule, which is readily crystallizable and can be subjected to X-ray crystallographic analysis; alternately, caseins can be studied using X-ray scattering technique [7].

Globular Milk Protein Structures

Globular proteins tend to be those that are soluble and consequently relatively easy to purify. Resolving the structure of β-lactoglobulin can help us understand the processes of denaturation and aggregation, which lead, for example, to heat exchanger fouling during milk processing [8].

Table 3: *Three-Dimensional Structures of Molecules Relevant to Dairy Science* [5].

Title	**Structure**	**Experimental data**	**Authors**
Chymosin complex with the inhibitor cp-113972 [10]		Resolution [Å]: 2.30 R-Value: 0.195 (work) R-Free: n/a Space group: H 3 2 Unit Cell: Length [Å] / Angles [°] a = 132.78 α = 90.00 b = 132.78 β = 90.00 c = 81.95 γ = 120.0	† Groves, M. R., † Dhanaraj, V., † Pitts, J. E., † Badasso, M., † Hoover, D., † Nugent, P., † Blundell, T. L.
Human α-lactalbumin, low temperature form [11]		Resolution [Å]: 1.15 R-Value: 0.122 (obs.) R-Free: 0.162 Space group: P 2_1 2_1 2_1 Unit Cell: Length [Å] / Angles [°] a = 33.19 α = 90.00 b = 49.55 β = 90.00 c = 64.20 γ = 90.00	† Harata, K., † Abe, Y., † Muraki, M.
Crystal structure of bovine α-lactalbumin [12]		Resolution [Å]: 2.20 R-Value: 0.216 (obs.) R-Free: 0.253 Space group: P 2_1 2_1 2 Unit Cell: Length [Å] / Angles [°] a = 72.04 α = 90.00 b = 104.65 β = 90.00 c = 117.42 γ = 90.00	† Chrysina, E. D., † Brew, K., † Acharya, K. R.
Crystal structure of apo-bovine α-lactalbumin [12]		Resolution [Å]: 2.20 R-Value: 0.191 (obs.) R-Free: 0.248 Space Group: P 4_1 2_1 2 Unit Cell: Length [Å] / Angles [°] a = 119.57 α = 90.00 b = 119.57 β = 90.00 c = 152.74 γ = 90.00	† Chrysina, E. D., † Brew, K., † Acharya, K. R.

Bovine β–lactoglobulin complexed with retinol, trigonal lattice [13]		Resolution [Å]: 2.40 R-Value: 0.210 (obs.) R-Free: 0.306 Space group: P 3_2 2 1 Unit Cell: Length [Å] / Angles [°] a = 53.58 α = 90.00 b = 53.58 β = 90.00 c = 110.94 γ = 120.00	† Kontopidis, G., † Sawyer, L.
Crystal structure of chloride saturated bovine lactoperoxidase at 2.5 A resolution shows multiple halide binding sites [14]		Resolution [Å]: 2.50 R-Value: 0.189 (obs.) R-Free: 0.219 Space Group: P 2_1 Unit Cell: Length [Å] / Angles [°] a = 54.44 α = 90.00 b = 80.56 β = 102.77 c = 77.71 γ = 90.00	† Singh, A. K., † Singh, N., † Sharma, S., † Kaur, P., † Singh, T.P.
β–lactoglobulin (native) [15]		Resolution [Å]: 2.10 R-Value: 0.216 (obs.) R-Free: 0.268 Space Group: C 2 2 2_1 Unit Cell: Length [Å] / Angles [°] a = 55.60 α = 90.00 b = 81.92 β = 90.00 c = 66.99 γ = 90.00	† Vijayalakshmi, L., † Krishna, R., † Sankaranarayanan, R., † Vijayan, M.
Crystal structure of c-lobe of bovine lactoferrin with dextrin at 1.9 Å resolution [16]		Resolution [Å]: 1.91 R-Value: 0.211 (obs.) R-Free: 0.241 Space Group: P 2_1 Unit Cell: Length [Å] / Angles [°] a = 61.81 α = 90.00 b = 50.13 β = 107.10 c = 65.54 γ = 90.00	† Mir, R., † Vikram, G., † Singh, N., † Sinha, M., † Sharma, S., † Kaur, P., † Singh, T. P.

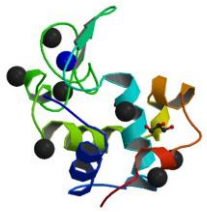
Crystal structure of hen egg white lysozyme [17]		Resolution [Å]: 1.40 R-Value: 0.194 (obs.) R-Free: 0.212 Space Group: P 4_3 2_1 2 Unit Cell: Length [Å] / Angles [°] a = 78.79 / α = 90.00 b = 78.79 / β = 90.00 c = 36.96 / γ = 90.00	† Abe, S., † Koshiyama, T., † Ohki, T., † Hikage, T., † Watanabe, Y., † Ueno, T.

Table 4: *Some Milk Proteins for which There Are Coordinate Data in the Protein Data Bank* [9].

Protein	Source	Method	Coordinates	Notes
Albumin	Human serum	X-ray	lao6, lbj5	Several species though coordinates not available
B-lactoglobulin [18]	Bovine	X-ray. NMR	lb0o, lbeb, lbso, lbsq, lexs, lcj5	Ovine, porcine, equine. Many more coordinate sets available
α-lactalbumin [18]	Buffalo	X-ray. NMR	l4v4, lalc, lb90, lhfx, lhmk, lhml	Several species. Goat recombinant protein.
Lactoferrin	Human	X-ray	lbol, lblx, lbma, lbiy, lcb6, llcf	Equine and buffalo also
Galactosyl transferase	Bovine	X-ray	lfg5	Catalytic domain expressed in tissue culture
Lactose synthase	Bovine	X-ray	lj8w	Atypical complex
Lipase	Cow bile	X-ray	lakn, laql	
Lysozyme	Echidna milk	X-ray	ljng, lqqy	Canine, also hen egg white
IgG_1	Human	X-ray	li4k, lcly	Fc-fragment complex
IgG_2	Mouse	X-ray	Ljbg	Fab fragment
IgA	Mouse	X-ray	2fbj	Fab fragment
IgM	Human	X-ray	Ladq	Complex with IgG fragment
β_2-Microglobulin	Bovine	X-ray	Lbmg	
Plasmin	Human	X-ray	Lbml	Catalytic domain complex with streptokinase
Xanthine oxidase	Bovine milk	X-ray	Lfiq	
Xanthine dehyrogenase	Bovine milk	X-ray	lfo4	

CONCLUSIONS

Production of well-ordered crystals and generating X-rays of suitable energy and wavelength are the two primary requisites of X-ray crystallography. This technique has widely been successfully used in elucidation of detailed three-dimensional structures of biological molecules, especially proteins.

X-ray crystallography has been used for analysis of liquid milk, milk powders, milk stones, polymorphism of milk fat and most widely and importantly in discovering the structure of most of the milk proteins and thus helping in correlating their structure with possible functions.

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