

A Maltose Biosensor for Determining Gelatinized Starch in Processed Cereal Foods

Emanuele Marconi,^{1,2} Maria Cristina Messia,¹ Giuseppe Palleschi,³ and Raimondo Cubadda¹

ABSTRACT

Cereal Chem. 81(1):6–9

A reliable method for the quantitative determination of gelatinized starch in processed cereal foods was developed. It consists of an electrochemical biosensor based on amyloglucosidase and glucose oxidase enzymes co-immobilized on a Pt electrode surface, and a third enzyme, α -amylase, added in solution. Analytical parameters such as time, temperature, and enzyme units were optimized. The degree of starch gelatinization was determined in different processed cereal foods using

the biosensor method and the results were commensurate to those obtained with the reference method. The biosensor methods showed good accuracy ($r^2 = 0.9629$; relative error <12%) and comparable precision (RSD <5%). This electrochemical system is rapid, reliable, inexpensive, user-friendly for unskilled operators, and can be a valid alternative to the methods traditionally used for gelatinized starch analysis.

Gelatinization is the transformation that occurs when a starch-water dispersion is heated. Above a certain temperature, the starch granules swell and their structure is altered (Leach 1965; Collison 1974; Greenwood 1976).

The gelatinization of starch is fundamental for many types of food production. Processes such as the baking of bread, the production of pasta products and starch-based snack foods, breakfast cereals, pregelatinized flour, baby foods, and parboiled cereals are all dependent on the proper gelatinization of starch to produce desirable technological, organoleptic, and nutritional properties in the end products (Olkku and Rha 1978; Lineback and Wong-srikasem 1980; Lund 1981).

The determination of the degree of starch gelatinization allows the assessment of starch susceptibility to hydrolysis and the relative metabolic response, understanding and control of the correctness of the process, the assessment of the influence of process and product variables, standardization and optimization of some of the operational conditions of extrusion cooking, and the assessment of cooking time and gelatinization temperature.

Degree of gelatinization can be determined qualitatively and quantitatively by physical, chemical, and biochemical methods such as loss of birefringence (Leach 1965), increase in viscosity (Sandstedt et al 1960), increase in enzyme susceptibility (Sullivan and Johnson 1964; Shetty and Lineback 1974; Kainuma 1994), decrease in enthalpy (Stevens and Elton 1971), proton magnetic resonance (Mendes et al 1996), loss of X-ray diffraction pattern (Collison 1968), and differential scanning calorimetry (Holm et al 1988; Marshall et al 1993). The most sensitive and most commonly used of these methods for quantifying gelatinization are the measurement of enzyme susceptibility and the loss of birefringence. All the above mentioned methods are often complicated or require expensive reagents, enzymes, and kits, or instrumentation and skilled operators. They do not satisfy the requirements for fast and simple measurements. The aim of this research was to develop a simple and reagentless electrochemical procedure based on the construction and assembling of an amperometric biosensor. Recently, as an alternative to the enzymatic-spectrophotometric methods, new sensing systems using biological molecules such as molecular recognition elements have been developed and applied to the measurement of polysaccharides and native enzymes in cereals such as starch, starch damage, β -glucan, and amylase

activity (Rennerberg et al 1983; Haginoya et al 1997; Marconi et al 1998; Boyaci et al 2002). In particular, our research group (Marconi et al 1998) standardized an amperometric system for the determination of damaged starch in flour and semolina.

In our study, this system was modified and improved to determine the degree of starch gelatinization in processed cereal foods. The principle of the method was the depolymerization of gelatinized starch into maltose and dextrans by α -amylase and the hydrolysis of these oligosaccharides into glucose by amyloglucosidase. Then glucose was oxidized to gluconic acid and hydrogen peroxide with glucose oxidase. The hydrogen peroxide produced in the last step was oxidized by an amperometric hydrogen peroxide platinum probe held at +650 mV (vs. Ag/AgCl). The output current was correlated to the glucose present in the sample, then to the amount of maltose, and finally to the gelatinized starch.

MATERIALS AND METHODS

Samples

Native starch and pregelatinized flours were supplied from Pavan Spa, Galliera Veneta, Padova, Italy. A series of samples containing different percentages of gelatinized starch (20, 30, 40, 50, 60, 80%) were prepared mixing different amounts of native starch with pregelatinized flour. In addition, various ($n = 25$) commercial processed cereal foods such as baby foods, pregelatinized flour and pastas, parboiled cereals, rusks, extrusion-cooked foods, and breakfast cereals were used for validating the method developed. The samples were ground in a refrigerated laboratory mill (model A10, Labortechnik, IKA, Staufen, Germany).

Materials

α -Amylase (type X-A: fungal, crude from *Aspergillus oryzae*, EC 3.2.1.1; 40 units/mg of solid), amyloglucosidase (AMG) from *Aspergillus niger* (EC 3.2.1.3; 51 units/mg of solid) for the biosensor method and from *Rhizopus mold* (EC 3.2.1.3; 20,800 units/g of solid) for the reference method, and glucose oxidase (GOD) from *A. niger* (type VII; EC 1.1.3.4; 176,000 units/g of solid) were from Sigma-Aldrich (St. Louis, MO). Immobilon AV affinity membrane TM 0.65- μ m pore size, 125- μ m thickness, was obtained from Millipore Corp. (Bedford, MA); cellulose acetate membrane (MW cut off \approx 100) was prepared in our laboratory according to Mascini et al (1987). Polycarbonate membrane, 0.03- μ m pore size, 6- μ m thickness, was obtained from Nucleopore (Pleasanton, CA). All other reagents were analytical grade from Sigma-Aldrich.

Apparatus

Electrochemical measurements were made with an ABD amperometric biosensor detector from Universal Sensors (Metairie,

¹ DISTAAM, Università del Molise, Via De Sanctis - 86100 Campobasso, Italy.

² Corresponding author. Phone: +39-0874-404616. Fax: +39-0874-404652. E-mail: marconi@unimol.it.

³ Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma "Tor Vergata" Via della Ricerca Scientifica - 00133 Roma, Italy.

LA). The electrochemical cell, consisting of a hydrogen peroxide probe, was from Universal Sensors. Currents were recorded with a Linseis L6512 recorder (Selb, Germany). Temperature studies were conducted using an ISCO GTR 90 thermostatic bath (ISCO Mi, Italy) with a wall glass beaker. Spectrophotometric measurements were made using a visible spectrophotometer (DMS 100S UV Varian Inc., Walnut Creek, CA).

Enzyme Immobilization Procedure

The Immobilon membrane was prepared by cross-linking the AMG and GOD enzymes with BSA and glutaraldehyde and then covalently binding them on a preactivated membrane following the procedure reported in previous work (Marconi et al 1998). In particular, 17 units of AMG, 17 units of GOD, 0.05 mg of BSA and 1 μ L of 0.25% glutaraldehyde were dissolved in 10 μ L of 0.5M KH_2PO_4 , pH 7.4, and placed on the membrane using a micropipette.

Probe Assembly

The maltose probe was assembled by placing membranes onto an inverted electrode jacket; these were a cellulose acetate membrane, an enzymatic membrane with immobilized GOD/AMG enzymes, and a polycarbonate membrane. The membranes were then secured to the electrode jacket with an O-ring. The jacket was filled with the appropriate electrode solution, and the combination working/reference electrode was inserted into the jacket and screwed into place.

Biosensor Procedure

Each sample was prepared in duplicate for the determination of gelatinized starch and total starch (determined as totally gelatinized starch). To determine the gelatinized starch fraction, an appropriate amount (20 mg) of ground sample was used, to which 5 mL of distilled water was added and thoroughly mixed, while for total starch determination, the following were added, in sequence, to another 20 mg of ground sample: 3 mL of distilled water, 1 mL of 1N NaOH, and after 5 min, 1 mL of 1N HCl. The sample was thoroughly mixed after each addition.

One mL (300U) of an α -amylase solution dissolved in a 0.1M; acetate buffer, pH 5.0, was then added to both samples. Each sample was immediately stirred on a vortex mixer for 5 sec and then incubated at 40°C for 30 min. The α -amylase was first dialyzed to remove the stabilizing sugars (glucose, maltose) present in the commercial enzyme, as stated by Haginoya et al (1997) and Marconi et al (1998). Then 10.0 mL of diluted sulphuric acid (0.2%, v/v) was added to terminate the reaction and the samples were filtered (Whatman No. 41).

Aliquots of the filtered samples (10 μ L) were added to 3 mL of acetate buffer, 0.1M, pH 5.0, where the electrode probe was equilibrated at 25°C. The change in current (nA), related to the H_2O_2 produced by untreated and completely gelatinized samples, was recorded after 3 min.

The current developed by the free glucose plus maltose present in the sample (blank) was determined after the addition of the diluted acid solution, instead of α -amylase, to stop the activity of endogenous enzymes. Then an aliquot of this solution was injected into the acetate buffer, where the probe was equilibrated and the free glucose (plus maltose) was measured.

The degree of starch gelatinization (DSG) of a sample is expressed as the percentage of gelatinized starch, as regards the completely gelatinized starch (total starch): $\text{DSG \%} = (\text{nA sample} - \text{nA blank}) / (\text{nA sample with completely gelatinized starch} - \text{nA blank}) \times 100$.

Reference Method

The reference method used was that of Chiang and Johnson (1977a,b), which is the one most often used for the quantitative determination of gelatinized starch in processed cereal foods, in both its original version (Varriano-Marston et al 1980) and modified versions (Lue et al 1991; Lin et al 1997). This method makes use of glucoamylase enzyme to convert gelatinized starch to glucose at 40°C in 30 min. The glucose produced reacts with *o*-toluidine reagent and then a spectrophotometric measurement of the color produced at 630 nm is taken.

RESULTS AND DISCUSSION

Gelatinized starch displays certain physicochemical properties resembling damaged starch. In particular, damaged and gelatinized starch are prone to enzymatic attack (Evers and Stevens 1985). Therefore, it should be possible to apply the same enzymatic principle to quantify both types of starch (Karkalas et al 1992). On the basis of these considerations, it was reckoned that a maltose biosensor, adjusted for the determination of damaged starch, could be adapted to the new requirements connected with the fact that the range of hydrolysable starch is far more extensive, varying from 0 to 100.

For these reasons, preliminary studies were made to identify the best operative parameters such as α -amylase units and the time and temperature of hydrolysis. The effects of α -amylase concentration on starch hydrolysis are shown in Table I. Variations in α -amylase concentrations, from 50 to 600 U/mL, caused significant changes in the rate of hydrolysis of susceptible starch. Table I illustrates that the conditions used for damaged starch analysis (50 U/mL of α -amylase at 40°C for 10 min) (Marconi et al 1998; AACC 2000) are not sufficient to obtain a complete hydrolysis of gelatinized starch. A maximum conversion of starch to maltose and then to glucose is provided using a minimum of 300 U/mL of α -amylase. In addition, when the same amount of α -amylase units

TABLE I
Degree of Starch Gelatinization of Pregelatinized Flour (with completely gelatinized starch) Determined by Amperometric Method with Different α -Amylase Units and Time and Temperature Combinations^a

α -Amylase Units	Temperature (°C)	Time (min)	Degree of Starch Gelatinization (%)
50	40	10	13.9
300	40	10	43.8
500	40	10	48.4
600	40	10	46.3
300	40	15	61.0
500	40	15	63.8
600	40	15	67.8
300	40	30	97.9
500	40	30	96.8
600	40	30	98.7

^a Results are the mean of two determinations.

TABLE II
Degree of Starch Gelatinization of Pregelatinized Flours (with completely and partially gelatinized starch) Determined by Amperometric Method Using 300 U/mL of α -Amylase and Different Time and Temperature Combinations^a

Temperature (°C)	Time (min)	Degree of Starch Gelatinization (%)	
		Pregelatinized Flour (DSG 100%)	Pregelatinized Flour (DSG 65%)
30	15	60.5	53.1
30	20	61.8	52.6
30	30	64.6	52.8
40	15	61.0	58.4
40	20	94.6	63.2
40	30	97.9	64.3

^a Results are the mean of two determinations.

TABLE III
Degree of Starch Gelatinization (%) of Flours with Known Amounts of Gelatinized Starch Determined by Amperometric and Reference Methods

Sample	Theoretical Value (T)	Reference Method (A)		Amperometric Method (B)		Relative Error (%)		
		Mean (n = 3)	RSD (%)	Mean (n = 3)	RSD %	T-A/T × 100	T-B/T × 100	A-B/A × 100
Mix 20	20	22.7	3.2	22.1	4.3	-11.9	-10.5	2.6
Mix 30	30	34.2	2.6	30.0	2.8	-14.0	0.0	12.3
Mix 40	40	37.1	4.5	37.8	1.3	7.2	5.5	-1.9
Mix 50	50	52.4	0.8	51.7	1.4	-4.8	-3.4	1.3
Mix 60	60	67.4	1.7	60.8	3.0	-12.3	1.3	9.8
Mix 80	80	81.4	1.6	80.2	1.1	-1.7	-0.2	1.5
PF 100	100	98.5	0.3	99.2	0.4	1.5	0.8	-0.7

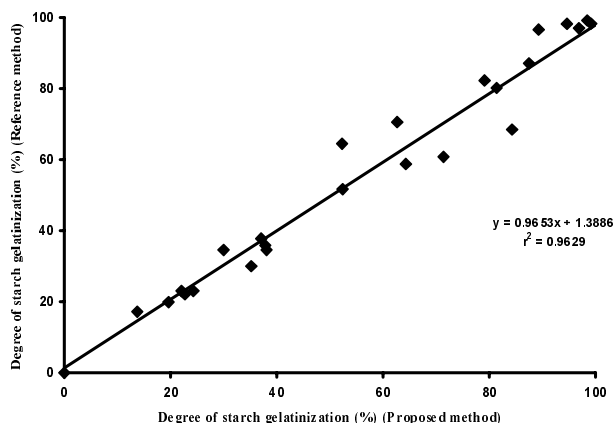


Fig. 1. Correlation between degree of starch gelatinization determined by proposed method and reference method in different processed cereal foods.

is used, time becomes an important variable for obtaining the complete hydrolysis of the gelatinized starch fraction, as is clearly shown in Table I. Therefore, a specific study on digestion time and temperature was conducted to define the best analytical conditions.

Table II reports the results obtained using two samples characterized by different percentages of gelatinized starch (100 and 65%, determined by the reference method), to verify the time and temperature conditions suitable for hydrolysis using 300 U/mL of α -amylase. As reported, when 40°C was applied for 30 min, the percentage of gelatinized starch obtained with the proposed method for both samples fitted very well (97.4 and 64.3%, respectively). For this reason, the best conditions selected for further studies were 300 U/mL of α -amylase, 30 min of incubation time, and 40°C temperature.

The accuracy of the proposed method was tested by using a series of samples containing known percentages of gelatinized starch, varying from 20 to 100%. Comparisons between the results obtained with the proposed and reference methods on samples with known percentages of gelatinized starch are reported in Table III. The relative error between two methods is good (from 1.9 to 12.3%). The accuracy of the amperometric procedure is better than the reference method when the theoretical value of the test samples is considered (relative error 0.0–10.5 vs. 1.5–14.0). The precision of the biosensor method is comparable to the reference method. In fact, the relative standard deviation (RSD) varies from 0.4 to 4.3 and from 0.3 to 4.5 for the biosensor and the reference method, respectively.

The biosensor procedure was further validated by assessing different cereal matrices (rusks, snacks, pregelatinized flours, baby foods, and extruded products) characterized by different amounts of gelatinized starch. The gelatinized starch content of different cereal products, determined by both the amperometric and reference method, are shown in Fig. 1. The regression equation was $y = 0.9653x + 1.3886$, with a correlation coefficient of $r^2 = 0.9629$. The findings show that the system is capable of analyzing

different kinds of products (different matrices) with a considerably wide range of gelatinization. In particular, DSG values <20% correspond to partially pregelatinized flours, DSG values varying from 40 to 90% include products such as rusks, breakfast cereals, baby foods, pregelatinized pastas, parboiled cereals, and extrusion-processed foods. DSG values >90% correspond to completely pregelatinized flours. Similar results were found by different authors (Lineback and Wongsrikasem 1980; Varriano-Marston et al 1980; Wootton and Chaudhry 1980; Holm et al 1988; Lue et al 1991; Karkalas et al 1992; Marshall et al 1993).

The complete time of analysis of the innovative procedure was 40 min versus 65 min of the reference procedure. Moreover, our method can be readily automated with a flow-injection apparatus (Haginoya et al 1997), making it highly suitable for a large number of tests. From an economical point of view, the biosensor method is more convenient than the reference procedures because it uses low amounts of reagents and enzymes, inexpensive instrumentation, and unskilled operators. In addition it is highly sensitive and selective without interferences from other reducing sugars or oligosaccharides, and it allows (using the same principle) determination of other compounds such as α -amylase, damaged starch, total starch, glucose, and maltose in flour, and processed cereal foods (Marconi et al 1998).

We can conclude that the hydrolysis of gelatinized starch with α -amylase solution, followed by the amperometric determination of maltose, provide a reliable technique for quantifying the amount of gelatinized starch in processed cereal foods.

ACKNOWLEDGMENTS

We would like to thank Claudio Pollini Pavan Spa for having supplied the samples of native starch and pregelatinized flours, and CNR (Consiglio Nazionale delle Ricerche) for the financial support.

LITERATURE CITED

- American Association of Cereal Chemists. 2000. Approved Methods of the AACC, 10th ed. Method 76-31. The Association: St. Paul, MN.
- Boyaci I. H., Şeker, U. O. Ş., and Mutlu, M. 2002. Determination of β -glucan content of cereals with an amperometric glucose electrode. *Eur. Food Res. Technol.* 215:538-541.
- Chiang, B. Y., and Johnson, J. A. 1977a. Measurement of total and gelatinized starch by glucoamylase and *o*-toluidine reagent. *Cereal Chem.* 54:429-435.
- Chiang, B. Y., and Johnson, J. A. 1977b. Gelatinization of starch in extruded products. *Cereal Chem.* 54:436-443.
- Collison, R. 1968. Swelling and gelation of starch. Pages 168-193 in: *Starch and Its Derivatives*. J. A. Radley, ed. Chapman and Hall: London.
- Collison, R., and Chilton, W. G. 1974. Starch gelation as a function of water content. *J. Food Technol.* 9:309-315.
- Evers, A. D., and Stevens, D. J. 1985. Starch damage. Pages 321-349 in: *Advances in Cereal Science and Technology*, Vol VII. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.
- Greenwood, C.-T. 1976. Starch. Pages 119-157 in: *Advances in Cereal Science and Technology*, Vol I. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.
- Haginoya, R., Sakai, K., Komatsu, T., Nagao, S., Yokoyama, K., Takeuchi, T., Matsukawa, R., and Karube, I. 1997. Determination of

- damaged starch and diastatic activity in wheat flour using a flow-injection analysis biosensor method. *Cereal Chem.* 74:745-749.
- Holm, J., Lundquist, I., Björk, I., Eliasson, A. C., and Asp, N. G. 1988. Degree of starch gelatinization, digestion rate of starch in vitro, and metabolic response in rats. *Am. J. Clin. Nutr.* 47:1010-1016.
- Kainuma, K. 1994. Determination of the degree of gelatinization and retrogradation of starch. Pages 137-142 in: *Carbohydrate Chemistry*, Vol X. J. N. BeMiller, ed. Wiley-Interscience: New York.
- Karkalas, J., Tester, R. F., and Morrison, W. R. 1992. Properties of damaged starch granules. I. Comparison of a micromethod for the enzymic determination of damaged starch with the standard AACC and Farrand methods. *J. Cereal Sci.* 16:237-251.
- Leach, H. W. 1965. Gelatinization of starch. Pages 289-307 in: *Starch: Chemistry and Technology*, Vol I. R. L. Whistler and E. F. Paschall eds. Academic Press: New York.
- Lin, S., Hsieh, F., and Huff, H. E. 1997. Effects of lipids and processing conditions on degree of starch gelatinization of extruded dry pet food. *Lebensm. Wiss. Technol.* 30:754-761.
- Lineback, D. R., and Wongsrikasem, E. 1980. Gelatinization of starch in baked products. *J. Food Sci.* 45:71-74.
- Lue, S., Hsieh, F., and Huff, H. E. 1991. Extrusion cooking of corn meal and sugar beet fiber: Effect on expansion properties, starch gelatinization, and dietary fiber content. *Cereal Chem.* 68:227-234.
- Lund, D. 1981. Influence of time, temperature, moisture, ingredients, and processing conditions on starch gelatinization. *CRC Crit. Rev. Food Sci. Nutr.* 20:249-273.
- Marconi, E., Baldino, C., Messia, M. C., Cubadda, R., Moscone, D., and Palleschi, G. 1998. Determination of damaged starch in wheat flour using an electrochemical biosensor maltose probe. *Anal. Lett.* 31:733-749.
- Marshall, W. E., Wadsworth, J. I., Verma, L. R., and Velupillai, L. 1993. Determining the degree of gelatinization in parboiled rice: Comparison of a subjective and an objective method. *Cereal Chem.* 70:226-230.
- Mascini, M., Mazzei, F., Moscone, D., Calabrese, D., and Massi-Benedetti, M. 1987. Lactate and pyruvate electrochemical biosensors for whole blood in extracorporeal experiments with an endocrine artificial pancreas. *Clin. Chem.* 33:591-593.
- Mendes da Silva, C. E., Ciacco, C. F., Barberis, G. E., Solano, W. M. R., and Rettori, C. 1996. Starch gelatinization measured by pulsed nuclear magnetic resonance. *Carbohydrates* 73:297-301.
- Oilku, J., and Rha, C. 1978. Gelatinization of starch and wheat flour starch. A review. *Food Chem.* 3:293-317.
- Rennerberg, R., Scheller, F., Riedel, K., Litschko, E., and Richter, M. 1983. Development of anti-interference enzyme layer for α -amylase measurement in glucose containing sample. *Anal. Lett.* 16:877-890.
- Sandstedt, R. M., Kemp, W., and Abbott, R. C. 1960. The effect of salts on the gelatinization of wheat starch. *Starch* 12:333.
- Shetty, R. M., Lineback, D. R., and Seib, P. A. 1974. Determining the degree of starch gelatinization. *Cereal Chem.* 51:364-375.
- Stevens, D. J., and Elton, G. A. H. 1971. Thermal properties of the starch/water system. I. Measurement of heat gelatinization by differential scanning calorimetry. *Starch* 23:8-11.
- Sullivan, J. W., and Johnson, J. A. 1964. Measurement of starch gelatinization by enzyme susceptibility. *Cereal Chem.* 41:73.
- Varriano-Marston, E., Ke, V., Huang, G., and Ponte, J. 1980. Comparison of methods to determine starch gelatinization on bakery foods. *Cereal Chem.* 57:242-248.
- Wootton, M., and Chaudhry, M. A. 1980. Gelatinization and in vitro digestibility of starch in baked products. *J. Food Sci.* 45:1783-1784.

[Received February 19, 2003. Accepted June 30, 2003.]