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Coagulation of Milk with Immobilized Proteases: A Review

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ABSTRACT

Enzymatic coagulation of milk by immobilized proteases, and their potential application to cheese manufacture, is reviewed. Particular emphasis is given to the immobilized protease catalyst and to the reactor design for coagulation of milk. Pepsin and chymotrypsin retained more activity and greater stability than the other immobilized proteolytic enzymes. Porous glass beads, several anion exchange resins, and the copolymer ethylene-maleic anhydride gave best results among the support materials that were evaluated. Covalent attachment of enzyme to support is preferable to adsorption techniques but may be too costly. Perhaps the best catalyst is one using a lengthy procedure for covalent immobilization of enzyme on glass beads but good results were also obtained with simpler adsorption techniques. Catalysts varied greatly in initial activity but all lost activity upon exposure to milk. Stirred tank, packed bed, and fluidized bed reactor designs were used. Continued research is required to make enzymic milk coagulation with immobilized proteases economically feasible.

A relatively new field, immobilized enzymes encompass surface and biological chemsitry, providing an interface for imaginative applications, be they theoretical or practical. Since about 1960 (*70*), immobilized enzymes have been discussed in over 1,500 research papers, many trade journal articles, numerous reviews (*1,50,61,65,73, 74,77,78,86-89,95*), several books (*40,57,68,100*), and various patents. Immobilized enzymes can offer certain advantages over soluble enzymes in areas such as the study of enzymes, analytical biochemistry, preparative pharmacology, and industrial processing — including food processing (Table 1). For example, in industrial processing, immobilized enzymes are reusable, generally more stable and more suited to continuous processing design than soluble enzymes. Specific uses of immobilized enzymes illustrating their wide variety of applications include a cobalamin binding study (*84*), elucidation of the casein micelle structure (*4, 15*), steroid transformation (*60*), leukemia therapy (*21*), urea-selective electrode (*39*), chill-proofing of beer (*96*), producing lactose-free milk (*25,98*), and reduction of nitrate and nitrate in waste treatment (*58*). Although immobilized enzymes have gained usage in a few commercial operations, there is considerable work remaining to fulfill their potential applications.

TABLE 1. *Soluble versus immobilized enzymes in food processing: Advantages and disadvantages*[1]

Disadvantages of soluble enzyme
1. Large amount of enzyme can remain in product to participate in further (undesirable) reaction.
2. Re-use of enzyme precluded.
3. Extent of reaction limited by product inhibition.
4. Precise control difficult.
5. If necessary, enzymatic reaction generally stopped by heating, which can be detrimental to the food as well as add another processing step.
6. Largely a batch process.

Advantages of immobilized enzyme
1. Enzyme reusable.
2. Reaction easily terminated by separating substrate from enzyme.
3. More precise control.
4. Less product inhibition.
5. Greater pH and temperature stability.
6. Can use enzymes presently unusable for various reasons.
7. Potential operation over greater pH range by modifying charge characteristics of support.
8. Continuous or batch use.
9. Greater reactor design flexibility.

Disadvantages of immobilized enzyme
1. Lower specific activity.
2. Inactivation with continued operation.
3. Cost of support and immobilization procedure.

[1]Advantages of the soluble enzyme are not listed. The soluble enzyme is the presently used form and thus implicitly has advantages.

An immobilized enzyme requires a support material, a method of immobilization, and an enzyme. A variety of enzymes have been immobilized (*100*). Support materials for enzyme immobilization are quite diverse (*73,74*) including: (*a*) *polymers* (*51*) such as polystyrene (*9, 33*), nylon (*30,37,44,59,81*), ethylene-maleic anhydride copolymer (*52*), and Teflon (*34*); (*b*) *inorganic substances* (*10,56,92*) such as alumina (*13*), glass (*72,83,90,93,94, 97*), stainless steel (*12,13,41*), and sand (*47,83*); (*c*) *natural products* such as cellulose (*49*), collagen (*22,35,45*), and chitin (*79,80*); and (*d*) *modified materials* such as DEAE-cellulose (*99,100*) and DEAE-Sephadex (*99,100*). The physical form of these supports is also quite diverse; they can exist as powders, particles, granules, sponges, gels, spheres, fibers, and membranes.

Several immobilization methods are available which may be placed in three categories: (*a*) *adsorption,* enzyme physically adsorbed to a water insoluble matrix; (*b*) *covalent attachment*, chemical bond formed between enzyme and support material; and (*c*) *entrapment*, chemical and/or physical localization of enzyme in water insoluble matrix. Examples from each category include physical adsorption of phosphatase on hydrophobic derivatives of cellulose (*11*), lactase covalently coupled to porous glass (*97*), and invertase entrapped in fibers (*53*).

Immobilized enzymes allow greater flexibility in reactor design than do soluble enzymes, which may be their most significant advantage. Common reactor designs for using immobilized enzymes are packed bed, fluidized bed, and stirred tank reactors (*5,8,27,50,89*). These reactors permit either batch or continuous processing but continuous processing is inherently more efficient than batch. Continuous operation would be the ideal usage of immobilized enzymes, particularly in food processing.

Many elegant covalent attachment and polymeric entrapment procedures using expensive, functionalized support materials have been published. Food processing with immobilized enzymes, however, probably will require simple immobilization procedures and inexpensive support materials for the foreseeable future. Unless exceptional performance is obtained with the more costly catalysts, the added expense of immobilization cannot be justified in a food processing system. Both commercially successful food processes (see below) using immobilized enzymes employ adsorption (a simple immobilization procedure) on inexpensive DEAE-modified matrices (*3,7,55,75,77*).

Thus, the ideal food processing application of immobilized enzymes will apparently require an inexpensive support, a simple preparation, and a reactor design permitting good substrate-enzyme contact in a continuous, free-flowing operation. Ultimately, use of immobilized enzymes will be an economic decision, based as much on catalyst cost and performance as on processing changes.

Examples of food processes that currently use immobilized enzymes are limited (*57,65,77,91*). However, immobilized enzymes are used as an integral part of two commercial food processing systems. The resolution of L-amino acids by immobilized L-amino acid acylase is a commercial process in Japan (*75,77*). The immobilized enzyme selectively removes the acetyl group from the optically active L-isomer of a racemic mixture of the acetylated amino acid. The resulting free L-form is easily separated from the acetylated D-form, which is then racemized chemically to regenerate more L-amino acid. Converting to this continous process from the previous batch operation reduced costs about 40%.

Glucose isomerase, one of the more important soluble enzymes in the food industry, is used in the other commercial immobilized enzyme system. Clinton Corn Processing Company received the 1975 IFT Food Technology Industrial Achievement Award for developing a process to produce high fructose corn syrup using immobilized glucose isomerase (*55*). Other companies—Novo Enzyme Corporation, Penick and Ford Corporation, and Corning Glass Works—have developed similar systems. The enzymatically converted cornstarch costs less than does invert sugar produced from sucrose, and has comparable sweetness. The commercial process involves liquefying raw cornstarch, saccharifying to dextrose, isomerizing to fructose, and refining (*3,7,77*). This is the first large scale commercial application of immobilized enzymes in a continous process in the world and is still the largest. Many other potential food processing applications of immobilized enzymes have been studied or proposed but none have become commercially viable (*57,65*).

COAGULATION OF MILK WITH IMMOBILIZED PROTEASES

Clotting milk with immobilized proteases may find application in cheesemaking (*48,66,67,71*). Current research in the cheese industry is attempting to make the traditional, labor-intensive, batch process more efficient and ultimately continous (*48,54,85*). Retaining the batch process, the industry has advanced through using larger batch units (*48*), thereby achieving an economy of scale. Mechanization (*64,85*)—and even automation (*63*)—of the various batch steps has also improved efficiency. New enzymes have been used to supplant the dwindling supply of rennet (*62*). Direct acidification has been researched as a way to avoid use of starter cultures (*69*).

A fully continuous cheesemaking system is still in the developmental stage. A continous process for making cottage cheese has been reported but is not being used (*28*). However, several commercial units are available that make individual batch steps continuous. For instance, equipment for continuous cheddaring and salting have been developed (*19,23,24,63*). A continuous coagulator using soluble milk clotting enzyme in a cold renneting step has also been reported (*48*).

Enzymatic milk coagulation, a two-phase process, involves first the enzymatic cleaving of a phenylalanyl-methionine bond in *k*-casein; this splits off negatively charged peptides and thus destabilizes the casein micelle (*29*). The secondary phase is physical aggregation of micelles to form a coagulum. The large temperature coefficient (Q_{10}) of the secondary phase but low Q_{10} of the primary phase permits separation of the two phases by lowering the temperature. Thus, the immobilized enzyme retains sufficient activity at lower temperatures to complete the primary enzymatic phase but clotting would not occur until after milk is removed from the immobilized enzyme and warmed.

Enzymatic coagulation of milk with immobilized proteases has been reviewed (*65,66,67,70,71*). This review is concerned with the potential application to cheesemaking of the coagulation of milk with the immobilized proteases reported in the literature. Thus, the following information about each previous research study is important and will be discussed: (*a*) enzyme used, (*b*) support used, (*c*) method of immobilization, (*d*) activity of resulting catalyst, (*e*) inactivation rate of catalyst, (*f*) reactor design, and (*g*) contact time and flow rate.

The various results reported in the literature are difficult to compare since they were obtained and reported in different ways. Most papers did not give enough information on which to compare activity on the basis of milligrams of bound enzyme. Furthermore, some researchers coagulated milk at its normal pH, some

acidified milk before enzyme contact, and others acidified milk after enzyme contact. Both phases of enzymatic milk coagulation are pH dependent—and the secondary phase is highly pH dependent—making results from these different pH values difficult to interpret. Reactor design, contact times, and flow rates varied but a reasonable idea of the immobilized protease activity and the milk clotting system was obtained from each paper and compared to the others.

Enzyme used

Use of immobilized instead of soluble enzymes for milk coagulation offers some advantages from the standpoint of the enzyme itself. First, there is a worldwide shortage of veal rennet, prompting use of other suitable proteases in traditional cheesemaking systems. However, the substitutes have some limitations (*62*). Shortage of milk-clotting enzymes may be eased by employing immobilized enzymes which can be re-used. Second, since the immobilized enzyme does not contaminate cheese, other proteases could be used that are not suitable in the soluble form because of excessive proteolysis. In addition, lack of contamination of cheese with milk-clotting enzymes would allow more controlled, longer ripening times. Greater storage stability would be advantageous with high-moisture cheeses since greater amounts of these types could be manufactured during peak milk production and held until seasonal slumps in production. In these types and other cheese varieties controlled amounts and types of proteases can be added to obtain the desired rate of proteolysis. Proteases can be selected for their beneficial effects on cheese ripening rather than a compromise between milk-clotting and cheese ripening capabilities. It may be possible also to infuse these proteases into cheese curd and eliminate their loss into cheese whey. While use of immobilized milk-clotting enzymes appears promising, yield, flavor, and texture of cheese made with immobilized clotting enzymes need to be researched.

Several proteolytic enzymes have been immobilized for use in milk coagulation (Table 2). Direct comparison of catalyst performance on the basis of enzyme immobilized is unfair since the support material and immobilization method have a great effect on catalyst activity. But, taking that into consideration, pepsin appears to be the best immobilized protease for coagulation of milk that is reported in the literature. Pepsin, an enzyme with a low pH optimum and not particularly active nor stable at the normal pH of milk, is quite active and stable upon immobilization (*14,16,18*). Perhaps this is due to the negatively charged supports to which it has been attached and which effectively put pepsin in a lower microenvironmental pH. Rennet was found to be more active but less stable than pepsin (*16*). It has been reported that chymotrypsin was used with some success (*99*). The *Mucor miehei* proteases, papain, and trypsin were not as effective as other milk-clotting enzymes used (*16,20,36*).

TABLE 2. *Proteolytic enzymes used in immobilized enzyme coagulation of milk*

Enzyme	References
Chymotrypsin	*16,26,38,99*
Mucor miehei proteases	*16*
Papain	*20,46*
Pepsin	*16,17,18,32,36,43*
Rennin	*16,38*
Rennet	*16,17*
Trypsin	*36*

Supports used

For coagulation of milk with immobilized proteases to be commercially feasible, the support material must be evaluated for cost, physical characteristics suitable for particular processing conditions, extent of enzyme-substrate contact, toxicity, and flow characteristics. A variety of support materials have been used to immobilize proteases for milk coagulation (Table 3). Milk-clotting enzymes have been adsorbed or covalently bound to several anion exchange resins (*38,39*). Three polymers—agarose, ethylene-maleic anhydride (EMA), and polyacrylamide—were used with EMA being most satisfactory (*20,36,38*). Porous glass beads were also used successfully (*16,17,32,43*); coating of the glass with zirconium oxide improved performance (*18*).

Methods of immobilization

Covalent attachment and physical adsorption are the two major methods reported in the literature for immobilizing milk clotting proteases (Table 3). No entrapment procedure has been published since this method is applicable only to small molecular weight substates able to diffuse in and out of the entrapping material.

TABLE 3. *Support materials and immobilization methods of immobilized proteases used in the coagulation of milk*

Support	Method of immobilization	References
Agarose	Covalent	*38*
EMA resin	Covalent	*36*
CM-cellulose	Covalent	*20,38*
Polyacrylamide	Covalent	*20*
Porous glass	Covalent	*16,17,32,43*
ZrO_2-coated porous glass	Covalent	*18*
DEAE-cellulose	Adsorption	*38,99*
Amberlite GC 400 I	Adsorption	*99*
DEAE-Sephadex	Adsorption	*99*
(Enzyme polymer)	Glutaraldehyde cross-linking	*46*

The method of immobilization is quite important in analyzing applicability of catalyst to commercial milk coagulation. Questions to be answered include: (*a*) Is the method simple and inexpensive? (*b*) Does the method prevent desorption of enzyme? (*c*) How much enzyme is bound? (*d*) What is the specific activity? (*e*) Will the method be approved for food use? Physical adsorption is the simplest, least expensive method but generally allows desorption of enzyme. Green and Cruthchfield (*38*) adsorbed rennin to DEAE-cellulose but found all of the resulting activity due to leached, soluble enzyme. However, Yanushauskaite et al. (*99*) adsorbed chymotrypsin first to a chlorotriazine dye and then to

anion exchange resins and reported no desorption of enzyme.

Covalent attachment of the milk-clotting enzyme to the support material has been used in most research. This procedure is preferable for laboratory studies but may be too complicated and costly for preparing immobilized cheesemaking enzymes. Covalent bonding usually eliminates desorption of enzymes; this problem was reported in one study but was subsequently corrected (*18*). Minimizing desorption of immobilized milk-clotting enzyme is important to lessen catalyst inactivation and control proteolysis in cheese curd during aging.

Although important for scale-up calculations, few papers report enzyme bound on a unit basis. Specific activity, also important, is inadequately reported. To illustrate the range reported, Yanushauskaite et al. (*99*) found retention of only 2-5% of the specific activity of the bound chymotrypsin compared to the soluble form whereas Goldstein (*36*) observed 40% retention of specific activity of pepsin upon immobilization. The ideal is to have a method producing a catalyst with maximum specific activity and activity per unit of support to minimize the size of the reactor.

Catalyst activity

Catalyst performance depends upon both activity and stability. A catalyst with high initial activity and a slow rate of inactivation is preferred. Comparison of catalyst activities reported in the literature is difficult since activity was determined in different ways. Activity depends on temperature of reaction, amount of enzyme used, pH of milk, contact time, etc., and these parameters varied among papers. However, initial activities varied greatly, suggesting real differences in the catalysts and not just in experimentation. For instance, Green and Crutchfield treated 5 ml of milk at 30 C with 1.7 mg of bound enzyme and observed clotting in 115 min. This low activity required a long contact time (flow rate of 240 ml/3 days) in their packed bed reactor. Cooke and Caygill (*20*) also reported low activity with a packed bed reactor, containing 15 mg of bound enzyme, operating at 4 C and a milk flow rate of 3-4 ml/h. Effluent milk clotted in 5 min after warming to 37 C. Yanushauskaite et al. (*99*) obtained somewhat better results. Milk (5 ml) treated for 15 min at 3 C with 20 mg of bound chymotrypsin clotted in 30 sec upon warming to 37 C.

Cheryan et al. (*14, 16-18*) obtained high activity with pepsin covalently coupled to porous glass beads. Milk of normal pH and at 10 C was passed through a fluidized bed reactor containing about 5 mg of bound enzyme. A contact time of only 1 min resulted in immediate clot formation after adjusting the milk to pH 6.1 and 30 C.

Inactivation rate

All previous reports indicated a decline in milk clotting activity of the immobilized enzyme during continuous exposure to milk. Although details of inactivation rates were given in only a few papers, it appears that these rates varied. Inactivation rates are very important for commercial operation; slow obviously is desirable. Inactivation seems to be a characteristic of all immobilized enzymes but is greatly accelerated when the substrate contains protein. Cooke and Caygill (*20*) found that repeated use of their immobilized papain led to a gradual loss in activity. Yanushauskaite et al. (*99*) observed that an initial flow rate of 3-4 ml/h produced a clot in 5 min but only five column volumes of milk later this flow had to be reduced to 1.5 ml/h to obtain the same clotting time. Ferrier et al. (*32*) observed a slower decline of milk-clotting activity of pepsin covalently attached to glass beads that was dependent upon pepsin source and temperature of milk being treated but independent of flow rate. Using the same catalyst, Cheryan et al. (*14, 16, 17*) obtained a slightly better stability of the immobilized protease activity by using milk of normal pH in a fluidized bed reactor rather than a packed bed reactor. Subsequently, Cheryan et al. (*18*) further improved stability of the immobilized protease activity and reported a 40-70-h useful catalyst lifetime in their system. They used a ZrO_2-coated controlled pore glass support and glutaraldehyde as the coupling agent to precoat the support with bovine serum albumin before attaching pepsin. The labile imine bonds formed between the proteins and glutaraldehyde during immobilization were reduced with sodium borohydride.

Reactor design

One of the great advantages in using immobilized proteases to coagulate milk is the flexibility in reactor design. The reactor is that vehicle providing contact between enzyme and milk (*50*). Three common designs—fluidized bed, packed bed, and stirred tank—which are applicable to continuous processing have been reported in the literature (Table 4). It is important that the reactor provide good enzyme-substrate contact but also allow free flow. Reactor design and the physical form of the catalyst must be complementary.

TABLE 4. *Reactor designs used in the coagulation of milk with immobilized proteases*

Reactor	References
Packed bed	*20,32,38,43*
Stirred tank	*26,36,38,99*
Fluidized bed	*16,17,18*

Both stirred tank and fluidized bed reactors appear well suited to treat milk with immobilized proteases. Shear forces in stirred tank reactors limit types of catalysts to those having sufficient structural integrity. The stirred tank can be used on a batch basis if the enzyme is filtered out after the reaction has been completed. The packed bed reactor, which has been investigated in many applications of immobilized enzymes, is limited by milk coagulating and plugging the column. The fluidized bed reactor alleviates plugging of the packed bed and can be used with supports that would disintegrate in a stirred tank (*8, 27*).

Contact time and flow rate

Contact time (with a known flow rate) determines reactor size and amount of immobilized enzyme required and influences the economics of a commercial continuous coagulator. A high flow rate and short contact time is desirable so that a large quantity of milk can be clotted by a small amount of catalyst in a small reactor.

Reported contact times varied greatly (Table 5). The extremely long contact times reported are not suitable for cheesemaking.

TABLE 5. *Contact times and flow rates used in the coagulation of milk with immobilized proteases*

Flow rate or contact time	Initial clotting time	Reactor temperature (C)	References
250 ml/3 days	115 min	4	*38*
3-4 ml/h	5 min	4	*20*
15 min	.5 min	3	*99*
1.7 min	1.7 min	36	*26*
1 min	immediate	10	*16,17,18,32,43*

CONCLUSIONS

A summary of the research literature concerning milk clotting with immobilized proteases is presented in Table 6. Of the immobilized proteases cited, the best system reported appears to be one using the catalyst and reactor design described by Cheryan et al. (*16, 18*). This system includes pepsin covalently attached to a zirconium oxide coated porous glass bead, precoated with bovine serum albumin. These beads are used in a fluidized bed reactor at low temperature to separate the two phases of enzymatic milk coagulation. Effluent milk is acidified and warmed for coagulation. Short contact times and high activity were observed. Inactivation of catalyst resulted in an approximately 50-h useful catalyst life.

However, this system is far from the ideal, and preliminary calculations show the system to be economically infeasible at present. The glass support material is costly, lacks sufficient density for optimum fluidization, and the immobilization procedure is lengthy. The clotting activity exceeds that of other reported immobilized proteases, but a catalyst with higher activity and a slower rate of inactivation would be much more desirable. Further studies on cheaper, denser supports and simpler immobilization procedures that give catalysts with comparable or greater activity are necessary to allow more favorable economic projections.

Milk is nearly an ideal food with which to study use of immobilized enzymes. It is a fluid and enzymes are already used in various milk processes (*2, 48, 76*). However, the protein in milk appears to deposit on the immobilized enzyme leading to a rapid decline in activity of the enzyme catalyst. Regeneration of the catalyst activity has been only partially successful (*14, 16, 32*).

Other immobilized enzymes have been studied for use in milk (*14, 48, 66, 67, 71, 76*). These include immobilized catalase to destroy hydrogen peroxide used in cold pasteurization of milk (*6*), immobilized peroxidases as antimicrobial agents (*66*), immobilized lactase to hydrolyze lactose (*94, 98*), and immobilized sulfhydryl oxidase to eliminate cooked flavor from milk sterilized by the Ultra-High Temperature process (*77, 82*). Perhaps use of immobilized proteases in a continuous system will eventually be integrated into commercial cheesemaking.

TABLE 6. *Coagulation of milk with immobilized proteases: A summary*

Enzyme	Support	Immobilization method	Activity	Inactivation rate	Reactor	Contact time	References
Chymotrypsin and rennin	Agarose	Covalent	Poor	—	Packed bed and stirred tank	Long	*38*
Rennin	DEAE-cellulose	Adsorption	Poor	—	Packed bed and stirred tank	Long	*38*
Papain	None	Glutaraldehyde cross-link	—	—	—	—	*46*
Pepsin and trypsin	EMA resin	Covalent	Good	Gradual	—	—	*36*
Papain	CM-cellulose and polyacrylamide	Covalent	Poor	Rapid	Packed bed	Long	*20*
Chymotrypsin	Amberlite and DEAE-cellulose and DEAE-Sephadex	Adsorption	Poor	—	Stirred tank	Medium	*99*
Chymotrypsin	CM-cellulose	Covalent	Poor	—	Stirred tank	Long	*26*
Pepsin	Porous glass	Covalent	Good	Gradual	Packed bed	Short	*32,43*
Rennin and chymotrypsin and *Mucor miehei* proteases	Porous glass	Covalent	Poor	—	Fluidized bed	Short	*16*
Pepsin	Porous glass	Covalent	Very good	Gradual	Fluidized bed	Short	*16,17*
Rennet	Porous glass	Covalent	Excellent	Gradual but faster than pepsin	Fluidized bed	Short	*16*
Pepsin	Porous glass	Covalent	Excellent	Slowest	Fluidized bed	Short	*18*

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REFERENCES

1. Ambrus, P. S. 1975. Insolubilized enzymes and other proteins. Review. J. Med. 6:217.
2. Arnold, R. G., K. M. Shahani, and B. K. Dwivedi. 1975. Application of lipolytic enzymes to flavor development in dairy products. J. Dairy Sci. 58:1127.
3. Aschengreen, N. H. 1975. Production of glucose/fructose syrup. Process Biochem. 10(4):17.
4. Ashoor, S. H., R. A. Sair, N. F. Olson, and T. Richardson. 1971. Use of a papain superpolymer to elucidate the structure of bovine casein micelles. Biochim. Biophys. Acta 229:423.
5. Atkinson, B. 1974. Biochemical reactors. Pion Limited, London.
6. Balcom, J., P. Foulkes, N. F. Olson, and T. Richardson. 1971. Immobilized catalase. Process Biochem. 6(8):42.
7. Barker, S. A. 1975. High fructose syrups—New sweeteners in the food industry. Process Biochem. 10(10):39.
8. Barker, S. A., A. N. Emery, and J. M. Novais. 1971. Enzyme reactors for industry. Process Biochem. 6(10):11.
9. Baum, G. 1975. Enzyme immobilization on macroreticular polystyrene. Biotechnol. Bioeng. 17:253.
10. Baum, G., and M. Lynn. 1975. Inorganic biomaterial supports. Process Biochem. 10(2):14.
11. Butler, L. G. 1975. Enzyme immobilization by adsorption on hydrophobic derivatives of cellulose and other hydrophilic materials. Arch. Biochem. Biophys. 171:645.
12. Charles, M., R. W. Coughlin, B. R. Allen, E. K. Paruchuri, and F. X. Hasselberger. 1973. Lactase immobilized on stainless steel and other dense metal and metal oxide supports. pp. 213-234. In R. B. Dunlap (ed.) Immobilized biochemicals and affinity chromatography. Plenum Publishing Co., N.Y., N.Y.
13. Charles, M., R. W. Coughlin, E. K. Paruchuri, B. R. Allen, and F. X. Hasselberger. 1975. Enzymes immobilized on alumina and stainless steel supports. Biotechnol. Bioeng. 17:203.
14. Cheryan, M. 1974. Applications of immobilized enzymes in milk systems. Ph.D. Thesis. University of Wisconsin, Madison.
15. Cheryan, M., T. Richardson, and N. F. Olson. 1975. Surface structure of bovine casein micelles elucidated with insolubilized carboxypepidase A. J. Dairy Sci. 58:651.
16. Cheryan, M., P. J. van Wyk, N. F. Olson, and T. Richardson. 1975. Continuous coagulation of milk using immobilized enzymes in a fluidized bed reactor. Biotechnol. Bioeng. 17:585.
17. Cheryan, M., P. J. van Wyk, N. F. Olson, and T. Richardson. 1975. Secondary phase and mechanism of enzymic milk coagulation. J. Dairy Sci. 58:477.
18. Cheryan, M., P. J. van Wyk, T. Richardson, and N. F. Olson. 1976. Stability characteristics of pepsin immobilized on protein-coated glass used for continuous milk coagulation. Biotechnol. Bioeng. 18:273.
19. Commonwealth Scientific and Industrial Research Organization, Australia. 1969. New cheese-making machine. Ind. Res. News 78: November 1969.
20. Cooke, R. D., and J. C. Caygill. 1974. The possible utilization of plant proteases in cheesemaking. Trop. Sci. 16:149.
21. Cooney, D. A. 1975. Biochemical and pharmacologic properties of L-asparaginase bonded to a dacron vascular prosthesis. Biochem. Pharmacol. 24:503.
22. Coulet, P. R., C. Godinot, and D. C. Gautheron. 1975. Surface bound aspartate aminotransferase on collagen films. Biochim. Biophys. Acta 391:272.
23. Czulak, J., and N. H. Freeman. 1967. A commercial continuous cheddaring machine. Aust. J. Dairy Technol. 22:6.
24. Czulak, J., N. H. Freeman, and A. L. Hammond. 1961. Mechanized cheese-making in commercial use. Dairy Eng. 78(2):58.
25. Dahlqvist, A., B. Mattiasson, and K. Mosbach. 1973. Hydrolysis of beta-galactosides using polymer-entrapped lactase. A study towards producing lactose-free milk. Biotechnol. Bioeng. 15:395.
26. Dolgikh, T. V., V. I. Surovtsev, L. V. Kozlov, V. K. Antonov, L. M. Ginodman, and V. I. Zvyagintsev. 1971. Investigation of the properties of chymotrypsin covalently bonded to carboxymethylcellulose in relation to clotting of milk. Prikl. Biokhim. Microbiol. 7:686.
27. Emery, A. N., J. S. Hough, J. M. Novais, and T. P. Lyons. 1972. Some applications of solid-phase enzymes in biological engineering. The Chem. Eng. 258:71.
28. Ernstrom, C. A., and C. G. Kale. 1975. Continuous manufacture of cottage and other uncured cheese varieties. J. Dairy Sci. 58: 1008.
29. Ernstrom, C. A., and N. P. Wong. 1974. Milk Clotting enzymes and cheese chemistry. pp. 662-771. In B. H. Webb, A. H. Johnson, and J. A. Alford (eds.) Fundamentals of dairy chemistry (2nd ed.), Avi Publishing Co., Westport, Conn.
30. Faulstich, H., A. Schafer, and M. Weckauf-Bloching. 1974. Alpha and beta galactosidases bound to nylon nets. FEBS Letters 48:226.
31. Ferrier, L. K. 1972. Possible uses of immobilized enzymes in the food industry. Ph.D. Thesis. University of Wisconsin, Madison.
32. Ferrier, L. K., T. Richardson, N. F. Olson, and C. L. Hicks. 1972. Characteristics of insoluble pepsin used in a continuous milk-clotting system. J. Dairy Sci. 55:726.
33. Filippusson, H., and W. E. Hornby. 1970. The preparation and properties of yeast beta-fructofuranosidase chemically attached to polystyrene. Biochem. J. 120:215.
34. Fishman, J. H. 1974. Polypeptide materials bound to fluorocarbon polymers. U.S. Patent 3,843,443. Oct. 22.
35. Giacin, J. R., J. Jakubowski, J. G. Leeder, S. G. Gilbert, and D. H. Kleyn. 1974. Characterization of lactase immobilized on collagen. Conversion of whey lactose by soluble and immobilized lactase. J. Food Sci. 39:751.
36. Goldstein, L. 1973. A new polyamine carrier for the immobilization of proteins. Water-insoluble derivatives of pepsin and trypsin. Biochim. Biophys. Acta 327:132.
37. Goldstein, L., A. Freeman, and M. Sokolovsky. 1974. Chemically modified nylons as supports for enzyme immobilization: Polyisonitrile-nylon. Biochem. J. 143:497.
38. Green, M. L., and G. Crutchfield. 1969. Studies on the preparation of water-insoluble derivatives of rennin and chymotrypsin and their use in the hydrolysis of casein and the clotting of milk. Biochem. J. 115:183.
39. Guilbault, G. C., and J. Montalvo. 1969. A urea-specific enzyme electrode. J. Amer. Chem. Soc. 91:2164.
40. Gutcho, S. J. 1974. Immobilized enzymes: Preparation and engineering techniques. Chemical Technology Review No. 39. Noyes Data Corp., Park Ridge, N. J.
41. Hasselberger, F. X., B. Allen, E. K. Paruchuri, M. Charles, and R. W. Coughlin. Immobilized enzymes: lactase bonded to stainless steel and other dense carriers for use in fluidized bed reactors. Biochem. Biophys. Res. Comm. 57:1054.
42. Hicks, C. L. 1974. I. Treatment of skimmilk and skimmilk fractions with immobilized pepsin. II. Oxidation of hydrocarbons by hemin. Ph.D. Thesis. University of Wisconsin, Madison.
43. Hicks, C. L., L. K. Ferrier, N. F. Olson, and T. Richardson. 1975. Immobilized pepsin treatment of skim milk and skim milk fractions. J. Dairy Sci. 58:19.
44. Hornby, W. E., and H. Filippusson. 1970. The preparation of trypsin chemically attached to nylon tubes. Biochim. Biophys. Acta 220:343.
45. Jakubowski, J., J. R. Giacin, D. H. Kleyn, S. G. Gilbert, and J. G. Leeder. 1975. Effect of calcium, magnesium and whey proteins on the activity of beta-galactosidase (*A. niger*) immobilized on collagen. J. Food Sci. 40:467.
46. Jansen, E. F., and A. C. Olson. 1969. Properties and enzymatic activities of papain insolubilized with glutaraldehyde. Arch. Bio-

chem. Biophys. 129:221.
47. Johnson, D. B., D. Thornton, and P. D. Ryan. 1974. Lactoperoxidase immobilization on inorganic supports. Biochem. Soc. Trans. 2:494.
48. Kosikowski, F. V. 1975. Potential of enzymes in continuous cheesemaking. J. Dairy Sci. 58:994.
49. Leemputten, E. V., and M. Horisberger. 1974. Immobilization of trypsin on partially oxidized cellulose. Biotechnol. Bioeng. 16:997.
50. Lilly, M. D., and P. Dunnill. 1971. Biochemical reactors. Process Biochem. 6(8):29.
51. Lindsay, A. S. 1969. Polymeric enzymes and enzyme analogs. J. Macromol. Sci.-Reviews Macromol. Chem. C3:1.
52. Lowenstein, H. 1974. The use of ethylene maleic anhydride for the preparation of a water-soluble polyanionic derivative of pepsin. Preparation and properties. Acta Chem. Scand. B28:1098.
53. Marconi, W., S. Gulinelli, and F. Morisi. 1974. Properties and use of invertase entrapped in fibers. Biotechnol. Bioeng. 16:501.
54. Maubois, J. L., and G. Mocquot. 1975. Application of membrane ultrafiltration to preparation of various types of cheese. J. Dairy Sci. 58:1001.
55. Mermelstein, N. H. 1975. Immobilized enzymes produce high-fructose corn syrup. Food Techno. 29(6):20.
56. Messing, R. A. 1974. Carrier for immobilized enzymes. Process Biochem. 9(9):26.
57. Messing, R. A., ed. 1975. Immobilized enzymes for industrial reactors. Academic Press, N.Y.
58. Mohan, R. R., and N. N. Li. 1974. Reduction and separation of nitrate and nitrite by liquid membrane-encapsulated enzymes. Biotechnol. Bioeng. 16:513.
59. Morris, D. L., J. Campbell, and W. E. Hornby. 1975. A chemistry for the immobilization of enzymes on nylon. The preparation of nylon-tube-supported hexokinase and glucose-6-phosphate dehydrogenase and the use of the co-immobilized enzymes in the automated determination of glucose. Biochem. J. 147:593.
60. Mosbach, K. 1973. Enzymes bound to artificial matrices. Sci. Amer. 224(3):26.
61. Mosbach, K. 1976. Immobilized enzymes. FEBS Letters 62 (Supplement):E80.
62. Nelson, J. H. 1975. Impact of new milk clotting enzymes on cheese technology. J. Dairy Sci. 58:1739.
63. Olson, N. F. 1970. Automation in the cheese industry. A review. J. Dairy Sci. 53:1144.
64. Olson, N. F. 1975. Mechanized and continuous cheesemaking processes for Cheddar and other ripened cheeses. J. Dairy Sci. 58: 1015.
65. Olson, N. F., and T. Richardson. 1974. Immobilized enzymes in food processing and analysis. J. Food Sci. 39:653.
66. Olson, N. F., and T. Richardson. 1975. Immobilized peroxidatic and milk-clotting enzymes. J. Dairy Sci. 58:1117.
67. Olson, N. F., and T. Richardson. 1974. Treatment of milk with immobilized proteases and oxidoreductases. pp. 329-336. In E. K. Pye and L. B. Wingard, Jr. (eds.) Enzyme engineering, Vol. 2. Plenum Publishing Co., N.Y.
68. Pye, E. K., and L. B. Wingard, Jr., eds. 1974. Enzyme engineering. Vol. 2. Plenum Press, N.Y.
69. Quarne, E. L., W. A. Larson, and N. F. Olson. 1968. Recovery of milk solids in direct acidification and traditional procedures of manufacturing pizza cheese. J. Dairy Sci. 51:527.
70. Richardson, T. 1974. Immobilized enzymes in food systems. Introduction. J. Food Sci. 39:645.
71. Richardson, T., and N. F. Olson. 1974. Immobilized enzymes in milk systems. pp. 19-40. In A. C. Olson, and C. L. Cooney (eds.) Immobilized enzymes in food and microbial processes. Plenum Publishing Co., N.Y.
72. Robinson, P. J., P. Dunnill, and M. D. Lilly. 1971. Porous glass as a solid support for immobilization or affinity chromatography of enzymes. Biochim. Biophys. Acta 242:659.
73. Royer, G. P., J. P. Andrews, and U. Rosa. 1973. Support materials for immobilized enzymes. Enzyme Technol. Dig. 3:99.
74. Royer, G. P., and W. E. Meyers. 1974. Support materials for immobilized enzymes and affinity chromatography. pp. 93-112. In E. Grushka (ed.) Bonded stationary phases in chromatography. Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan.
75. Sato, T., T. Mori, T. Tosa, I. Chibata, M. Furui, K. Yamashita, and A. Sumi. 1975. Engineering analysis of continuous production of L-aspartic acid by immobilized *Escherichia coli* cells in fixed beds. Biotechnol. Bioeng. 17:1797.
76. Shipe, W. F., E. C. Lee, and G. F. Shenyk. 1975. Enzymatic modification of milk flavor. J. Dairy Sci. 58:1123.
77. Skinner, K. J. 1975. Enzymes technology. Chem. Eng. News 53 (33):22.
78. Stanley, W. L., and A. C. Olson. 1974. The chemistry of immobilizing enzymes. J. Food Sci. 39:660.
79. Stanley, W. L., G. G. Walters, S. H. Kelly, B. G. Chan, J. A. Garibaldi, and J. E. Schade. 1976. Immobilization of glucose isomerase on chitin with glutaraldehyde and by simple adsorption. Biotechnol. Bioeng. 18:439.
80. Stanley, W. L., G. G. Walters, B. Chan, and J. M. Mercer. 1975. Lactase and other enzymes bound to chitin with glutaraldehyde. Biotechnol. Bioeng. 17:315.
81. Sundarum, P. V., and W. E. Hornby. 1970. Preparation and properties of urease chemically attached to nylon tube. FEBS Letters 10:325.
82. Swaisgood, H. E., V. G. Janolino, and P. S. Patrick. 1975. Immobilization of sulfhydryl oxidase and some of its kinetic properties. J. Dairy Sci. 58:796. (Abstr.).
83. Thornton, D., A. Francis, D. B. Johnson, and P. D. Ryan. 1974. The immobilization of lactoperoxidase and beta-fructofuranosidase on glass and on sand, by the metal-link method. Biochem. Soc. Trans. 2:137.
84. Toraya, T. 1975. Immobilized diol dehydrase and its use in studies of cobalamin binding and subunit interaction. Biochemistry 14:4255.
85. Trauberman, L. 1975. Cheesemakers reap dividends with mechanized systems. Food Eng. 47(6):59.
86. Vieth, W. R., and K. Venkatasubramanian. 1973. Enzyme engineering. Part I. The utility of supported enzyme systems. Chemtech 3:677.
87. Vieth, W. R., and K. Venkatasubramanian. 1974. Enzyme engineering. Part II. Materials for immobilized enzyme reactors. Chemtech 4:47.
88. Vieth, W. R., and K. Venkatasubramanian. 1974. Enzyme engineering. Part III. Properties of immobilized enzyme systems. Chemtech 4:309.
89. Vieth, W. R., and K. Venkatasubramanian. 1974. Enzyme engineering. Part IV. Process engineering for immobilized enzyme systems. Chemtech 4:434.
90. Weetall, H. H. 1969. Alkaline phosphatase insolubilized by covalent linkage to porous glass. Nature 223:959.
91. Weetall, H. H. 1975. Immobilized enzymes and their application in the food and beverage industry. Process Biochem. 10(6):3.
92. Weetall, H. H., and R. A. Messing. 1972. Insolubilized enzymes on inorganic materials. pp. 563-595. In M. L. Hair (ed.) The chemistry of biosurfaces. Vol. 2. Marcel Dekker, Inc., N.Y.
93. Wierzbicki, L. E., and V. H. Edwards. 1973. Immobilization of microbial lactases by covalent attachment to porous glass. J. Food Sci. 38:1070.
94. Wierzbicki, L. E., V. H. Edwards, and F. V. Kosikowski. 1974. Hydrolysis of lactose in acid whey by lactase bound to porous glass particles in tubular reactors. J. Food Sci. 39:374.
95. Wingard, L. B., Jr., ed. 1972. Enzyme engineering. Biotechnol. Bioeng. Symp. No. 3 Interscience Publishers, John Wiley and Sons, N.Y.
96. Witt, P. R., R. A. Sair, T. Richardson, and N. F. Olson. 1970. Chillproofing beer with insoluble papain. Brewers Dig. 45(10):70.
97. Woychik, J. H., and M. V. Wondolowski. 1972. Covalent bonding of fungal beta-galactosidase to glass. Biochim. Biophys. Acta 289:347.
98. Woychik, J. H., and M. V. Wondolowski. 1973. Lactose hydrolysis

in milk and milk products by bound fungal beta-galactosidase. J. Milk Food Technol. 36:31.
99. Yanushauskaite, V. B., L. V. Kozlov, and V. K. Antonov. 1974. Milk-clabbering and proteinase activity of modified alpha-chymotrypsin sorbed on anionites. Prikl. Biokhim. Microbiol. 10:410.
100. Zaborsky, O. R. 1973. Immobilized enzymes. CRC Press, Cleveland, Ohio.