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Ü. LILLE

APPLICATION OF PROSTAGLANDIN SYNTHETASE IN PREPARATIVE OXIDATION OF POLYENIC ACIDS TO PROSTAGLANDIN DRUGS *

Biochemical synthesis was the first and simplest process for the preparation of prostaglandins (PG) needed in increasing quantities for biological studies after the elucidation of their structure in the early sixties [1, 2]. Soon the limitations of the biochemical process (low capacity, incompatibility with analog synthesis) were understood and synthetic activities were directed towards semisynthetic and total chemical preparation [3]. With the aid of the latter, at the beginning of the seventies the commercial production of PG began to correspond to the growing demand for biological studies, human and veterinary medicine and animal breeding. Up to now, more than twenty drugs [4, 5] have been registered on the basis of PG and their analogs used in million doses.

Meanwhile research work on the characterization of PG synthetase was continued, and from time to time attempts were made to increase the capacity of the biochemical process [6–8]. Attention was also paid to biosynthesis using microorganisms [3, 9, 10].

In this paper we have summarized the results of the research work aimed at developing high-capacity biochemical PG synthesis and assessing future prospects.

Our attention was mainly devoted to PGE, particularly to PGE_2. PGE can be easily converted chemically to PGF of high yields and further to PGI.

The total efficiency of biochemical synthesis depends on the characteristics of the biocatalyst and reaction system, resources of enzyme, parameters of preparative process, and the existence of proper drug forms. Let us focus our attention upon those items.

Characterization of biocatalyst and reaction system. The formation of PG is catalyzed by at least two enzymes, PGH synthetase (dioxygenase) and PGH convertase (PGE isomerase, etc., Table 1). The rate-limiting enzyme, dioxygenase, is a unique enzyme catalyzing oxidation and reduction in one sequence of molecular changes of the fatty acid substrate. It is a membrane-bound hemeprotein consisting of two polypeptide chains with a molecular mass of about 70 kD. The heme is reversibly bound to an apoenzyme. The enzyme activity depends on the S—S bonds, serine-like OH groups and an imidazole ring of histidine. These characteristics and also the spectral data are consistent with typical hemeproteins. Unlike hemoglobin, the holoenzyme is insensitive to carbon monoxide.

* Lecture held at the second All-Union conference «Synthetic and applied studies of prostaglandins», Ufa, October 1984.

Table 1

Characterization of PG synthetase [11–19]*

PGH synthetase (EC 1.14.99.1) isolated from sheep vesicular glands	
yield, % of activity	10—30
purification ratio (-fold)	>65
polypeptide chain molecular mass, kD	70
quantity of chains	2
sequence of amino acids	DAERAAOPEAPAPV
quantity of hemes per chain	1
Heme-apoferment complex	
K_D, M	10^{-7}
E_{412}, $mM^{-1} \cdot cm^{-1}$	120
E_{280}, $mM^{-1} \cdot cm^{-1}$	130
K_D CN, M	10^{-2}—10^{-4}
CO	insensitive
Concentration of activator (ROOH), M	10^{-6}—10^{-7}
Sensitivity to dithiothreitol + N-ethylmaleimide	sensitive
aspirin	"
glutaric aldehyde	"
diethylpyrocarbonate	"
Dioxygenase activity, μm of acid/mg·min	35
optimal pH	7—8
Michaelis constant (K_M), M	
for arachidonic acid	10^{-5}—10^{-6}
for O_2	$5 \cdot 10^{-6}$
for hydroquinone	$5 \cdot 10^{-5}$
k_{cat}/k_{in} (for acid)	10^2—10^3
K_J (8,12,14-eicosatrienoic acid), M	10^{-7}
Peroxidase activity	
μm of H_2O_2/mg·min	3
PGE isomerase (EC 5.3.99.2) isolated from bovine vesicular glands	
yield, % of activity	6—7
coenzyme	GSH
activity, μm/mg·min	2

* For detailed literature, see [11, 17].

The oxidation of fatty acid substrate is a free radical reaction, being similar to that of plant lipoxygenases. The specificity for polyenic acid with the methylene-interrupted *cis*-double-bond system is considerably high (Table 2). The C_{20} (C_{19}) w 6 acids are strongly favoured. The methyl branch interferes with the PG-formation, and this effect depends on the location of the branch in the chain. Chemically various groups in the α position to the carboxyl group inhibit the conversion of the substrate more strongly than the methyl group. It is accepted that the fatty acid substrate is bound to the enzyme before oxygen, the second substrate. This is the case for cytochrome P-450 as well [24].

In the reductive step, a third substrate is needed, viz. the electron donor. The specificity of the donor is very low: biologically active amines, phenols, inorganic salts, etc. act almost similarly. The structure of the co-oxidation products is donor-dependent.

The mechanism of the dioxygenase reaction seems to be parallel to that of autoxidation [11]. The main role of the enzyme is to ensure the translocation of the side chains of endoperoxide [25] and an approximately 10^5-fold acceleration of the reaction.

The characteristic feature of dioxygenase is its self-destruction during catalysis [26]. This phenomenon belongs to mechanism-based enzyme inactivations [27], and there is no effective way of avoiding this process entirely.

Various approaches have been proposed for regulating dioxygenase activity in the living cell. The question has not been solved as yet, and

Table 2

Substrate specificity of PG synthetase [20–23]

Precursor	Conversion, %	Precursor R-20:3 w 6	Conversion, %
18:3 w 4	—	2-Me	70
10:3 w 6	7	3-Me	35
18:4 w 6	15	5-Me	48
19:3 w 5	2	10-Me	10
19:3 w 6	85	13-Me	3
19:4 w 5	44	17-Me	12
19:4 w 6	58	18-Me	9
20:3 w 5	3	19-Me	20
20:3 w 6	96	2-COOH	1
20:3 w 7	25	2-$COOCH_3$	14
20:3 w 8	—	2-$COOC_7H_5$	14
20:3 w 9	—	2-CH_2OH	13
20:4 w 6	100	2-C_4H_9	19
20:5 w 3	7	2-NH_2	1
21:3 w 6	51	2-F	33
21:3 w 7	30	2-Br	37
21:4 w 6	35	2-CN	28
21:4 w 7	52	2-CN-2-C_4H_9	14
22:3 w 6	7	2-Ph	24
22:3 w 8	—	2-$NHCOCH_3$	3
22:4 w 6	34	2-OCH_3	24
		2-CH_2OCH_3	35
		Δ^2-*cis*	52
		Δ^2-*trans*	35
		3,3-di-Me	28
		4,4-di-Me	1

it is not known whether the inactivated dioxygenase in the living cell is «wounded» or «dead».

Resources of dioxygenase. The dioxygenase is a key enzyme in the biochemical and semisynthetic PG synthesis. By the way, the endoperoxide formation with proper stereochemistry from simple reagents is so far beyond any synthetic method.

During the recent 20 years no great success has been achieved in increasing the resources of PG endoperoxide synthetase. The seminal vesicles of ovines and bovines remain the most accessible raw materials. Therefore the use of DNA recombinant methods for cloning endoperoxide-synthetase-producing microorganisms is highly desirable.

In relevant literature, there is strong evidence of PG synthesis in plants [28–30]. This is probably the result of the action of various plant lipoxygenases on the acid substrate. If a suitable type of lipoxygenase should be found, then the cultivation of proper plant cells might yield

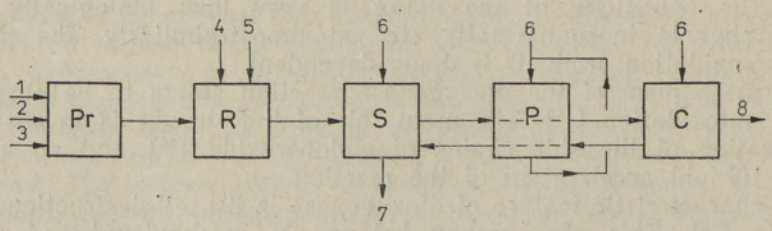

Fig. 1. Main steps of biochemical synthesis of PG. Pr — preparation, R — incubation, S — separation, P — purification, C — concentration. *1* — vesicular glands, *2* — buffer, *3* — cofactors, *4* — fatty acid substrate, *5* — air, *6* — solvents, *7* — wastes, *8* — product.

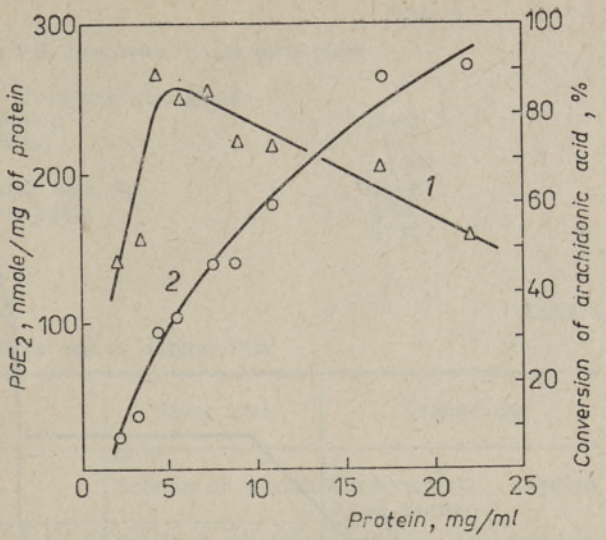

Fig. 2. Specific yield of PGE_2 (*1*) and degree of conversion of arachidonic acid (*2*) vs. protein concentration.

a catalyst for biochemical oxidations (or direct biosynthesis could be performed).

Parameters of the preparative process. The most important features in designing a biochemical process seem to be the proper biocatalyst, its working conditions, and possibilities for product separation and purification (Fig. 1).

The dioxygenase activity determines the product concentration, and the latter together with the physical state of catalyst is decisive in the subsequent separation procedure.

In modern enzyme technology enzyme immobilization is widely used. In many cases it stabilizes the catalyst and enables recycling. Nowadays one can find many examples of immobilization for all classes of enzymes to various carriers, including oxygenases and PG-dioxygenase [8]. So far there is, however, no case of successful protection against suicidal inactivation. As long as the self-destruction of the biocatalyst is not markedly decreased, the use of immobilized dioxygenase is economically inacceptable. In this case the use of synthetase in the form of tissue homogenate with a specific activity of about 0.1 μmole arachidonic acid/mg protein min is advantageous. The recycling of microsomal protein [6] appeared to be far less favourable.

The most important characteristics of the reactor are product capacity per unit of reactor volume, specific yield of the product and the degree of conversion of the fatty acid substrate. As can be seen from Fig. 2, a compromise between the values of these parameters is needed. It is to be pointed out that for each protein concentration there exists an optimal concentration of the fatty acid substrate for obtaining maximal specific yield. For the homogenate protein it is about 0.4—0.5 μmole/mg.

It should be borne in mind that the preparative synthesis conditions are quite different from those used for the in vitro enzyme studies. The latter system is usually homogeneous, and the concentration of organic components is on a micromole level or lower. There is enough oxygen dissolved in the reaction medium equilibrated with air for a reaction to occur. The preparative conversion of the fatty acid substrate takes place in a multiphase system, and the concentration of most components is on a millimole level. Therefore the recommendations made on the basis of measurements in a polarographic unit or a spectral cuvette must be checked very carefully until any statement about pre-

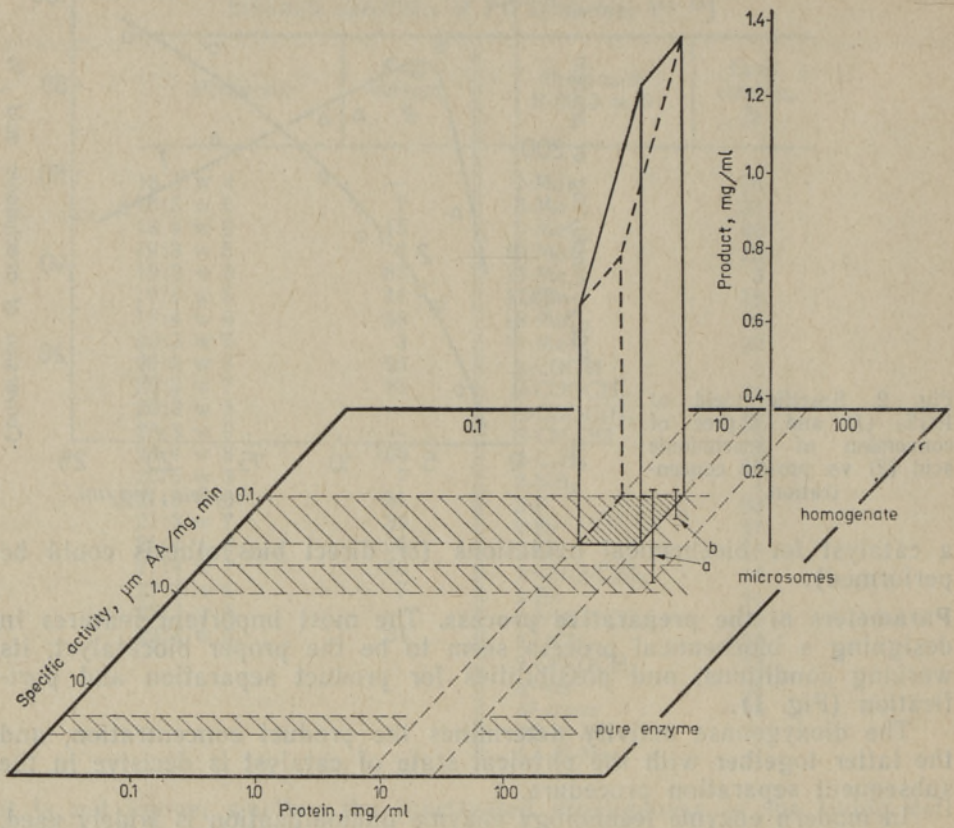

Fig. 3. Choice of biocatalyst and volume capacity of reactor. Specific activity (μmole arachidonic acid/mg protein min) and protein concentration (mg/ml) vs. product concentration (mg/ml). The cut rectangle shows parameters of our process, *a*, *b* — according to [3,6].

parative process can be made. This concerns the use of organic solvents or solutions with a high ionic power. From this point of view, preparative studies [3, 6] played an important role in further development. However, the product yields (mainly PGE_2) remained low (below 0.3 mg/ml).

This is why a reinvestigation of all parameters of the system, including cofactors, nonionic detergents and organic solvents, was undertaken. After that, the dispersity of the system was guaranteed.

Due to these studies, the PGE_2 yield of about one mg/ml was obtained in reactor volume (Fig. 3).

On the present technological level, separation of protein from the reaction mixture remains critical for further dilution of the reaction system. Conventional precipitation with organic solvents results in a further 2—3-fold increase in volume followed by a strictly controlled evaporation of the solvent added. For further product purification one-step chromatography and crystallization are quite sufficient. Some characteristics of the process are given in Table 3.

In fact, it is one-pot synthesis, and in a 10-litre working-volume reactor one can obtain amounts of PGE_2 (or E_1) on a kg level per year.

During the recent six years, this process has been in use for the production of natural PGs for biological and medical studies [31], and in a near future its whole capacity will be used to produce PGs for wider use in medicine.

Table 3

Data on the PG production in the pilot plant

Product yield, g/kg of sheep vesicular glands	
in solution of incubation	3—4
crystalline PGE_2 (95%)	2.5
ratio k_{cat}/k_{in}	>1000
Conversion of arachidonic acid, %	38—46
Reagents and materials, kg/kg	4 300
Wastes, kg/kg	2 750

Table 4

Clinical use of natural PGs

PG	Drug	Drug form	Present use
E_1	Alprostadil	Solution in alcohol	Cardiovascular system, blood storage
E_2	Dinoprostone (Prostin E_2, Minprostin, Prostarmon E, Prostenon)	Solution in alcohol, tablets	Obstetrics and gynecology, gastrointestinal disorders, bronchospasm, urinary retention
$F_{2\alpha}$ THAM salt	Dinoprost (Prostin $F_{2\alpha}$, Amoglandin, Minprostin $F_{2\alpha}$, Prostarmon F, Prostamodin F)	Saline solution	Obstetrics and gynecology, urinary retention
I_2 Na salt	Epoprostenol sodium (Cyclo-Prostin)		Cardiovascular system, asthma, gastrointestinal, ocular and renal disorders, gynecology

Clinical use and drug forms. The clinical use of basically studied natural PGs as drugs has been generally accepted [4, 5] and the main fields of their use are presented in Table 4.

The wide range of pharmacological effects and chemical and metabolic instability are the main disadvantages of natural PGs, and there are already about 25 selected analogs for present or potential use [5]. However, studies are being conducted on the pharmaceutical preparation of drugs and ways of administration of natural PGs. Instead of a systemic administration, the local one is used, which culminates in the viscous stabile gel of PGE_2 for terming labour induction [32]. In case of local use, a 5- to 10-fold decrease in dose and a high specificity of action without any side effects is achieved. Furthermore, suitable solid carrier materials for molecular encapsulation have been found to stabilize the unstable PGE_2 (and I_2) for storage at room temperature. Therefore in certain cases the natural PG drugs are competitive with those prepared on the basis of analogs (for example, the PGE_2 gel for cervix ripening [32, 33]).

In cooperation with pharmacologists of Tartu State University we have succeeded in developing the drug «Prostenon» on the basis of PGE_2 for introduction and stimulation of labour. Successful clinical trials have been performed in seven clinics (about 800 patients). At present this preparation is being clinically tested for treating renal disorders, asthma and hypertension.

Chemical modification and preparation of derivatives of natural PGs for a potentiation of their biological effects is likewise promising [3, 34].

Conclusion

The appearance of natural PG drugs competitive with those prepared on the basis of analogs has increased the importance of biochemical (bio)synthesis among PG synthetic ways.

At the present technological level with a sufficient enzyme supply, the biochemical synthesis supplemented by chemical methods permits a simple way of preparing natural PGs and their modifications for wide use in certain fields of medicine.

Future research in the whole complex PG area will yield further success in biochemical PG synthesis and wider use of properly modified natural PGs.

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Academy of Sciences of the Estonian SSR,
Institute of Chemistry

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O. LILLE

PROSTAGLANDIINIDE PREPARATIIVNE BIOKEEMILINE SÜNTEES JA RAVIMITE VALMISTAMINE

Artiklis on vaadeldud prostaglandiinide (PG) sünteesi polüeenhapete biokeemilise oksüdatsiooni teel ja ravimite valmistamise võimalusi looduslike PG-de baasil ning analüüsitud andmeid biokatalüsaatori ja reaktsiooni süsteemi kohta, osalevate ensüümide ressursse, preparatiivse protsessi parameetreid ja looduslike PG-de ravivorme ning rakendusi. On järeldatud, et biokeemilise sünteesi lihtsus ja sobivate ravivormide olemasolu loob eeldused looduslike PG-de (eriti PGE_2) laiaks rakenduseks meditsiinis, eeskätt günekoloogias.

Ю. ЛИЛЛЕ

ПРИМЕНЕНИЕ ПРОСТАГЛАНДИН-СИНТЕТАЗЫ ДЛЯ ПРЕПАРАТИВНОГО ОКИСЛЕНИЯ ПОЛИЕНОВЫХ КИСЛОТ И ПОЛУЧЕНИЕ ЛЕКАРСТВ НА БАЗЕ ПРОСТАГЛАНДИНОВ

В статье рассматриваются состояние препаративного биохимического синтеза простагландинов (ПГ) и возможности изготовления лекарственных средств на базе природных ПГ. Параметрами, определяющими эффективность процесса в целом, являются свойства биокатализатора, условия препаративного процесса, ресурсы ПГ-синтетазы и наличие подходящих лекарственных форм. Простота биохимического синтеза и стабильность лекарственных форм создают предпосылки для широкого применения природных ПГ (особенно $ПГЕ_2$) в медицине, в первую очередь в гинекологии.