Scale-up of Enzymatic Solid-to-Solid Peptide Synthesis and Enzyme Recovery

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The feasibility of scale-up for applications of enzymatic solid-to-solid synthesis of dipeptides was investigated by using Thermoase catalyzed synthesis of Z-Asp-Phe-OMe as a model reaction. The reaction system involves typically only 10% (w/w) enzyme suspension added to dry solid substrates. Enzymatic solid-to-solid biocatalysis combines the main advantages of aqueous media, high catalytic activity, low water media, and high yields. A flow sheet for the whole process is described, as well as the use of a jacketed reactor with an anchor stirrer for various steps of the process, including the neutralization reaction to adjust the acid-base condition of the reaction mixture. A solution for enzyme recycling, an important issue for scale-up applications, is also suggested.

Introduction

There is a rapidly growing market for enantiomerically pure compounds, which are used as active ingredients or as a basis for powerful new drugs. The production scale of some products is at the boundary between fine and bulk chemicals. The dipeptide sweetener aspartame, with an annual consumption of around 9,000 metric ton in 1992 in the United States alone, is one of the most commercially successful examples, and the world-wide demand is still expected to rise at an annual rate of 5.5% (Bizzari et al., 1996). Enzymatic solid-to-solid synthesis, a new approach offering yields of 600 mg product per g initial reaction mixture, can be seen as a useful tool in the transition from synthesis of fine to bulk chemicals (for a review, see Erbeldinger et al., 1998b). The synthesis of the aspartame precursor Z-Asp-Phe-OMe is also one of the most widely used model reaction systems in enzymatic dipeptide synthesis; it is an obvious choice to study the feasibility of scaling-up enzymatic solid-to-solid synthesis.

The origin of enzymatic solid-to-solid synthesis dates back to two independent studies in the early 1990s (Kuhl et al., 1992, and Gill and Vulfson, 1993). The term "solid-to-solid synthesis" refers to a reaction mixture of solid substrates with added liquid (often pure water) around 10% (w/w). The reaction takes place in the liquid phase, which is saturated with substrates. With the product precipitating rapidly during formation, the overall visual impression of the reaction mixture is a solid or highly viscous suspension. An equilibrium of 95% product is possible (Halling et al., 1995; Eichhorn, 1995), even though the reaction occurs in an aqueous reaction medium, which is normally associated with low equilibrium yields. The product makes up a very high proportion of the total reaction mixture in comparison with dissolved reaction systems, which typically have yields below 10 mg product per g reaction mixture. In addition, the absence of any organic solvents is an advantage, especially in regard to food and pharmaceutical industries.

Initial investigations of solid-to-solid synthesis at a preparative scale have been published by Kuhl et al. (1995) and Eichhorn et al. (1997). They reported the application of a

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fluidized bed and a dough mixer on a scale as high as 3 g-moles. These reactors were needed to deal with the problems of mixing the sticky and viscous reaction mixture. The present article investigates further some features that would be important for the feasibility of an industrial-scale operation of enzymatic solid-to-solid synthesis. We examine key steps in a likely flow sheet with a view to scale-up. Furthermore we propose a method for enzyme recovery and recycling. We examine the neutralization of a substrate supplied in the form of a hydrochloride salt, and use an industrial-grade enzyme instead of a pure one.

Material and Methods

Enzymes and chemicals

Thermoase PS 160, an industrial grade thermolysin, was purchased from Daiwa Kasei K.K., Osaka, Japan. Carbobenzoxy-L-aspartic acid (Z-L-Asp) and L-phenylalanine methyl ester hydrochloride salt (L-Phe-OMe·HCl) were obtained from Bachem, UK. The $KHCO_3$ and Hammarsten casein were of an analytical grade from BDH, UK.

The mixer

After various attempts, we decided that the optimal design on scale-up should be a type of double sigma blade mixer, as these continuously scrape sticky substances off the walls, and the mixer blades themselves are counterrotating and self-scraping. The mixer used in our scale-up study consists of two concentric stainless-steel beakers of a volume of 500 mL and 750 mL, respectively (Figure 1). The gap between the beakers was welded. Two stainless-steel tubes were also incorporated for the purpose of temperature control. A Perspex lid containing a closable sample port was used to seal the contents within the mixer. The impeller chosen is an anchor blade driven by a Heidolph RZR1 overhead motor (Torque of around 100 Nm at a rotation speed of 40 rpm). To prevent the reaction mixture from sticking on the anchor blade, we mounted two internal baffles on the Perspex lid to achieve a scraping effect.

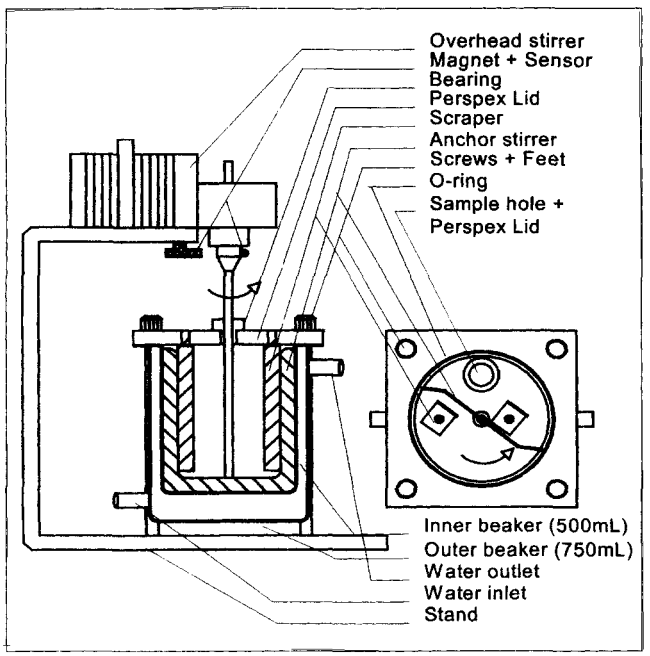

Figure 1. Mixer used for neutralization, solid-to-solid reaction, and product washing.

Ultrafiltration module

To concentrate the enzyme solution we used ultrafiltration in an Amicon Stirred Cell Unit (8400) from Millipore, UK. The volume is 400 mL, and the minimum final volume after concentration is 10 mL. This unit itself comprises a magnetic stirrer and an ultrafiltration membrane disk from Millipore UK (Amicon YM 10,000NMWL, regenerated cellulose, with a diameter of 76 mm). The unit operates under 4.5 bar pressure. For a 19-h process over three days, we stored the entire ultrafiltration device at 4°C overnight in order to minimize enzyme inactivation.

The reaction procedure

The standard reaction procedure used in the scale-up study was similar to our previous Z-Gln-Leu-NH_2 reactions at a 2-mg·mol scale (Erbeldinger, 1998a). An additional step was implemented to control the acid–base condition of the reaction mixture. Before the start of the enzymatic reaction the solid starting materials were mixed in a mol ratio of 1.0 Z-Asp, 1.0 Phe-OMe·HCl, and 1.5 $KHCO_3$. The neutralization reaction was initiated by adding 100 mL H_2O per mol substrates and mixing the reactor contents for about 2 min to release the CO_2 produced. The resulting highly viscous mixture was kept for one hour at room temperature prior to the addition of the enzyme. Additional mixing was necessary from time to time in order to control foaming. After that the industrial grade Thermoase powder (150 g enzyme per mol substrates) was added and the mixture was briefly mixed for about 1 min, then the reaction mixture was incubated at 40°C. Depending on the reaction scale and the aims of the experiment, the reaction mixture was then

1. Distributed to eppendorf tubes as described in Erbeldinger et al. (1998a) (2-mmol scale for initial rate experiments)
2. Transferred to a 7-mL or 20-mL vial (2 mmol or 20 mmol scale for washing experiments)
3. Kept in the mixing vessel (100 mmol and 200 mmol scale).

The eppendorf tubes and the reaction vials were completely immersed in a water bath, whereas the mixing vessel was jacketed.

HPLC analysis

The reaction was terminated by dissolving solid samples into a liquid mixture (50% v/v) of aqueous 20 mM H_3PO_4 and acetonitrile. A Gilson 715 HPLC system with a reversed-phase Hichrom column (HIRPB-250A, 0.46×25 cm) was used for analysis at a flow rate of 1.0 mL min^{-1} and detection at 257 nm. The mobile phase consists of 60% (v/v) acetonitrile mixed with an aqueous phase containing 2 g L^{-1} triethylamine adjusted to a pH of 2.5 with H_3PO_4.

Hydrolytic enzyme assay of Thermoase PS160

For the hydrolytic enzyme assay a Radiometer pH stat apparatus (ABU80 autoburette and PHM82 pH meter) controlled by a microcomputer was applied. The substrate solution contained 20-g L^{-1} Hammarsten casein and 10 mM $CaCl_2$, adjusted with 1 M NaOH (about 15 mL per L solution) to a pH of 8.0. Each assay was carried out using 5-mL substrate solution and 100 mM NaOH as titrant, at a pH of 8.0 and a controlled temperature of 25°C. To allow for variations in the substrate solution, a calibration assay using freshly dissolved Thermoase powder (100 mg crude enzyme powder per mL water) was performed each day. Activity measurements were usually presented as an equivalent mg Thermoase PS160, or compared within experiments as a percentage of the initial activity. The standard Thermoase PS160 calibration gave an alkali consumption of around 50 μmol min^{-1} mg^{-1}. For comparison thermolysin gave an alkali consumption of around 340 μmol min^{-1} mg^{-1}, and therefore has an activity that is about seven times higher than that of the industrial-grade enzyme preparation. Using the same assay, Cassells and Halling (1988) estimated that these values were equal to a bond cleavage rate of 100 μmol·min^{-1}·mg^{-1} and 680 μmol·min^{-1}·mg^{-1}, respectively. Samples were diluted to yield rates between 1 and 5 μmol min^{-1} in order to show a linear dependence on the amount of enzyme. Compared to thermolysin, Thermoase is about 20 times less active in a biphasic system of water and *n*-butyl acetate (Hirata et al., 1997), and about 10 times less active in a enzymatic solid-to-solid reaction (Erbeldinger et al., 1999).

To measure the remaining enzyme activity during the synthesis reaction, we separated a solid sample (around 0.1 mmol) from the solid reaction mixture, and dissolved it completely in deionized water (15 mL) prior to assay. The measurement of activity was normalized against the weight of the sample.

Results and Discussion

Enzymatic solid-to-solid synthesis of Z-aspartame

A key feature of enzymatic solid-to-solid synthesis is the fact that the reaction takes place in a liquid phase saturated with both substrates. The model reaction was Z-Asp and Phe-OMe·HCl, leading to the synthesis of the aspartame precursor, Z-Asp-Phe-OMe. Our results showed high yields of around 80% conversion to product with high substrate concentrations [around 10% (w/w) added water], leading to a very efficient synthesis methodology. The results of our work on the synthesis of Z-aspartame allow a direct comparison with other popular enzymatic reaction methods available (see Table 1 for such an overview).

The results for the solid-to-solid synthesis (Nos. 15 and 16) clearly stand out in comparison with other methods. The main

Table 1. Principal Methods for Thermolysin-Catalyzed Synthesis of Z-Asp-Phe-Ome

No.	Researchers	System Temp. If Not Stated ≈ 40°C	Z-Asp/Phe-OMe Ratio*	Rate nmol·min^{-1}·mg^{-1}	Yield %
1	Murakami et al. (1996)	Aqueous	1:0.82	735‡	4.7
2a	Inouye (1992)	Aqueous	1:1	83.1‡‡	3.0
2b		+2.73 M NaCl	1:1	232‡‡	2.0
3a	Hwang et al. (1995)	H_2O sat with EtAc**	1:1.5	168‡	7.7
3b		H_2O/EtAc 50/50% (v/v)	1:1.5	36.7‡	35.2
3c		EtAc sat with H_2O	1:1.5	20.0‡	58.0
4a	Miyanaga et al. (1995)	tAmOH†	1:5	4.33‡,#	95***
4b		25/75 tAmOH/EtAc	1:5	8.42‡,#	95
4c		25/75 tAmOH/EtAc+4% H_2O	1:5	1.9‡,#	99
5a	Ooshima et al. (1995)	EtAc/benzene/methanol/H_2O	1:2	3.0‡	—****
5b		(50:29:19:2)			
6a	Nagayasu et al. (1994a)	EtAc+3.5% (v/v) H_2O	1:1	1.0‡,#	—****
6b			1:5	7.9‡,#	
7	Nagayasu et al. (1994b)	tAmOH+4% (v/v) H_2O	1:1.7	1.7§§,#	87
8	Nakanishi et al. (1985)	EtAc plug-flow reactor	1:2.5	1.8§§,#	95
9	Nakanishi et al. (1990)	EtAc plug-flow reactor 25°	1:5	1.0§§,#	97
10	Murakami and Hirata (1997)	Aqu./BuAc†† biphase low pH extractive	1:16	20.0§§,##	97
11	Isono et al. (1995)	Hollow-Fiber w solv. extractive Aqu.+BuAc phase	1:1.5	1.9§§	91
12	Isono et al. (1997)	Aqu./BuAc biphase perstractive	1:8	35.0§§	—****
13	Hirata et al. (1997)	Biphase extractive reactor Aqu/BuAc	1:15	20.2§§,##	94
14	Persichetti (1995)	CLECs EtAc 55°C	1:3	26.7§,‖	100
15	Eichhorn et al. (1997)	Solid-to-solid+2.2 mol NaOH	1:2	429‖‖	>90.0
16a	Erbeldinger et al. (1999b)	Solid-to-solid+2.5 mol $KHCO_3$	1:1	390‡	≈80
16b		+1.5 mol $KHCO_3$	1:1	140‡	≈90

*[mol mol^{-1}].
**EtAc = ethyl acetate.
***Continuous process.
****Product extracted from organic phase, no overall yield.
†tAmOH = *tert*-amyl alcohol.
††BuAc = *n*-butyl acetate.
‡Initial rate.
‡‡Rate calculated using the time required to reach half of the equilibrium yield.
§Thermolysin CLECs (cross-linked enzyme crystals, rate per mg protein.
§§Continuous reaction, rate calculated using yield and residence time.
‖Rate calculated using yield at 20 h.
‖‖Rate calculated using yield at 7 h.
#Immobilized Thermolysin (rate per "wet protein," rate per mg dry protein must be higher).
##Thermoase PS160 used as catalyst (rate corrected by a factor of 20 to give catalytic rate equivalent to thermolysin).

advantages of the enzymatic solid-to-solid peptide synthesis can be seen in the high productivity due to the high concentration of substrate and therefore of product. The absence of organic solvents in the reaction mixture is also an advantage. From the viewpoint of the overall reaction mixture, solid-to-solid synthesis occurs in a low-water medium, that is, the overall water content can be as low as 10% (w/w), and the product precipitates out. The comparison between aqueous and organic solvents in Table 1 is in line with common perceptions of enzymatic catalysis. The aqueous methods offered the highest initial rate, but with the lowest equilibrium yield (Nos. 1, 2, and 3a). The low water/organic solvent media were the opposite, having high equilibrium yields but lower catalytic rates (Nos. 3b to 14). Note that the commercial TOSOH process used on a large scale by the Holland Sweetener Company for the synthesis of Z-aspartame was not included in Table 1, even though it achieved product yields above 95% (Schoemaker et al., 1997; Harade et al., 1997). This is because the process was based on a unique precipitation phenomenon that is not suitable as a model for other dipeptides.

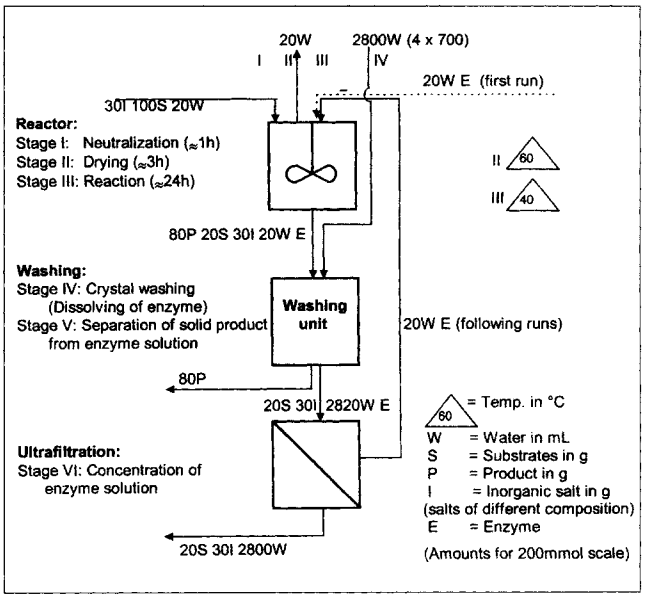

Figure 2. Flow sheet of enzymatic solid-to-solid peptide synthesis with integrated enzyme recycling.

Scale-up of solid-to-solid synthesis with integrated enzyme recycling

Most other methods shown in Table 1 provided some kind of enzyme recycling, for example, the use of immobilized enzyme (Nos. 6 to 9 and 14), or of free enzymes dissolved in an aqueous phase with the product extracted from a separate organic phase (Nos. 11 to 13). However, there has been no previous attempt at enzyme recycling after solid-to-solid synthesis in a reactor on our scale. As the term solid-to-solid synthesis indicates, the enzyme is trapped among product crystals at the end of the reaction, so recovery might be a complex process.

The flow sheet proposed in Figure 2 illustrates how enzyme recovery and recycling could be accomplished in solid-to-solid synthesis on scale-up. Following our previous kinetic investigations (Erbeldinger et al., 1998a, 1999b), the flow sheet also incorporates the use of Phe-OMe in its hydrochloride salt form, and an industrial source of thermolysin called Thermoase PS160. Since the price per unit activity of Thermoase is significantly cheaper than the commercially available pure thermolysin, it would be important for the viability of scaled-up applications. As shown in Figure 2, the process for enzymatic solid-to-solid peptide synthesis with integrated enzyme recycling contains several stages.

Stage I. Initial dry mixing of starting materials, followed by mixing in the water to start the neutralization of the reaction mixture.

Stage II. Drying of the reaction mixture to remove water.

Stage III. Enzymatic solid-to-solid reaction, started by blending with enzyme suspension.

Stage IV. Washing of the product crystals to recover enzyme and to remove substrate and salts.

Stage V. Separation of product crystals from enzyme solution.

Stage VI. Concentrating enzyme solution as required for Stage II, via an ultrafiltration process.

Stage I needs a stirring mechanism that is capable of solid mixing, and temperature control (such as water jacket). The possibility of supplying dry air through the reaction mixture is necessary in Stage II. Stage III requires both temperature control and mixing in order to blend highly concentrated enzyme suspension with dried solid substrates, giving a viscous paste. In Stage IV the purpose of the mixer is to break aggregates of product crystals in order for the trapped enzyme to be released and dissolved in the washing liquid. At this stage the system consists of a liquid with suspended product crystals, and the process is similar to liquid mixing. The separation of the product and the concentration of the enzyme solution are carried out in two separate stages, using a centrifuge or filter (Stage V) and ultrafiltration device (Stage VI).

Stage I: neutralization

Neutralization incorporates the initial mixing of the substrates. At a 200-mmol (0.2-gmol) scale, the mixer designed for this study proved to be able to provide efficient mixing of dry solid substrates and inorganic basic salts, as indicated by the HPLC analysis of the substrate ratio in samples. The mixer also successfully blended water with the solids, which yielded a viscous suspension. The neutralization stage is necessary to adjust the acid-base condition of the reaction mixture, for example, by shifting the pH closer to the optimum for the subsequent enzymatic catalysis. Details of the large effects on reaction rate are described elsewhere (Erbeldinger et al., 1999b). During the neutralization reaction, the release of CO_2 produced foam. At a rotational speed of 40 rpm or more in our mixer, the foaming can be minimized.

Stage II: drying

Drying prior to the reaction is an essential process that permits enzyme recycling. The water content of the reaction mixture is an important reaction parameter that mainly influences the reaction yield and the reaction rate (Erbeldinger et

Table 2. Drying Methods Prior to Thermoase PS160 Catalyzed Synthesis of Z-Aspartame*

Drying Method	Time h	Water Removal, %	Subsequent Enzymatic Reaction: Initial Rate nmol·m^{-1}·mg^{-1}	Subsequent Enzymatic Reaction: Yield %
N_2 gas stream at 40°C	5.5	84	15.0	85
N_2 gas stream at 60°C	3.0	100	12.8	85
Vacuum evaporator at 90°C	2.0	100	< 1.0	N/A

*Two mmol Z-Asp, 2 mmol Phe-OMe·HCl, 3 mmol $KHCO_3$, 300 mg Thermoase P160, and 200 μL H_2O per reaction. Reactions were started by adding water and enzyme powder after the drying process was completed.

al., 1998a). In our system the overall water concentration should not exceed a range of 10 to 20% (w/w). Since this amount of water is added in the enzyme suspension that results from the ultrafiltration process, the rest of the reaction mixture has to be dry at this stage. Any standard drying process should be suitable. After the neutralization reaction, we placed the reaction vial horizontally into a heating unit, and pumped N_2 over it, which proved to be sufficient (Table 2). Reactions performed in this way achieved identical catalytic rates and yields to our previous method, which involved adding solid enzyme to the wet substrate mix. As an alternative, we tried a vacuum evaporator at 90°C, which provided complete drying within 2 h (whereas N_2 drying took 3 h at 60°C and more than 5.5 h at 40°C). After drying at 90°C, however, there was a significant decrease in the initial rate. The reason for this was probably chemical decomposition of the substrates, as shown, on HPLC analysis, by the appearance of new peaks.

In small-scale reactions the recovered enzyme solution can be freeze-dried, making both the drying stage and enzyme ultrafiltration unnecessary. However, freeze-drying would probably not be economically viable at a larger scale.

Stage III: reaction

The solid-to-solid synthesis requires an initial mixing of substrates and enzyme, and the control of the reaction temperature. These are fairly simple to scale-up, as the reaction mixture initially consists of a mainly liquid suspension with intermediate viscosity. As the reaction proceeds, further mixing could be used, either continuously or intermittently. In this case, the mixer has to cope with an increase in apparent viscosity due to the precipitation of product. Beyond 40% conversion, the reaction mixture becomes a highly viscous paste rather than a liquid. The paste needs to be continuously scraped from the walls of the mixer body and the scraper itself. As a result, scraping is an essential feature that needs to be included in any mixer for solid-to-solid synthesis. Eichhorn et al. (1997) described the successful use of a dough mixer on a 3-mol scale, containing two blades mounted on a single rotating axis and scraping the walls of the mixer body.

In order to investigate the effect of mixing on reaction rate and yield, we performed a reaction (100 mmol) with continuous mixing throughout. The initial rate was 15.6 nmol min^{-1} mg^{-1}, which is no different from our tests at 2 mmol scale. Due to the distinct brown color of the enzyme suspension, it was easy for us to observe that the initial blending process was usually completed within half a minute. When the viscosity increased with product formation, this had a detrimental effect on the mixing process. After around 2 h, two large masses of paste were visible, one stuck to the anchor blade and the other on the fixed scraper. In light of this, it is not surprising that mixing has no effect from this stage onwards, and also that there was no difference in the final yield as compared with the standard unmixed procedure. It is clear that it will be very difficult for any mixer design to bring about motion on the microscopic scale throughout the reaction mixture. Without this, any improvement of mass transfer and of the overall reaction performance is highly unlikely. In conclusion, our mixer was very successful for the initial mixing of the substrates and enzyme suspension.

As a result of this experiment, we thought that operating the impeller of our reactor might have no benefit beyond the initial mixing. We therefore carried out an experiment where we mixed only on the following schedule: (a) initial mixing of dry substrates (2 min); (b) during the neutralization reaction (about 1 h); and (c) after adding the enzyme powder (2 min). We obtained initial rates of 14.5 ± 0.5 nmol product per min per mg Thermoase and final yields of $80.7 \pm 4.3\%$ product conversion. These were identical to our results on the smaller scale of 2 nmol. During the reaction, samples were taken from different locations at the top, middle, and bottom of the reactor. We found that samples from the top surface of the reaction mixture showed up to 8% less product conversion in comparison with samples taken elsewhere. The lower product conversion can be explained by the observation of condensed water droplets on the lid of the reactor (which was not temperature controlled), which then fell onto the surface of the reaction mixture. As described previously (Erbeldinger et al., 1998a), an increase in the water concentration led to a decrease in reaction performance. To eliminate this problem, we placed the complete reactor in a temperature-controlled cabinet, and found that a standard deviation of under 1% between samples was achieved. It is now clear that a fully jacketed reactor is sensible in further applications of enzymatic solid-to-solid synthesis.

If the biocatalyst is to be recovered and recycled, it is important to know the loss of enzymatic activity during the reaction period. Figure 3 shows this for two different levels of $KHCO_3$ addition. As discussed previously (Erbeldinger et al., 1999b), an increase in the amount of $KHCO_3$ added caused an increase in the initial rate, but a decrease in the final yield. Hence we studied stability at two different levels of $KHCO_3$ addition. The relatively high scatter in Figure 3 results from the problems of sampling the heterogeneous reaction mix, and is typical for solid-to-solid reactions (Erbeldinger et al., 1998a). Nevertheless, it is clear that Thermoase is relatively stable in standard reaction conditions, with 1.5 mol $KHCO_3$

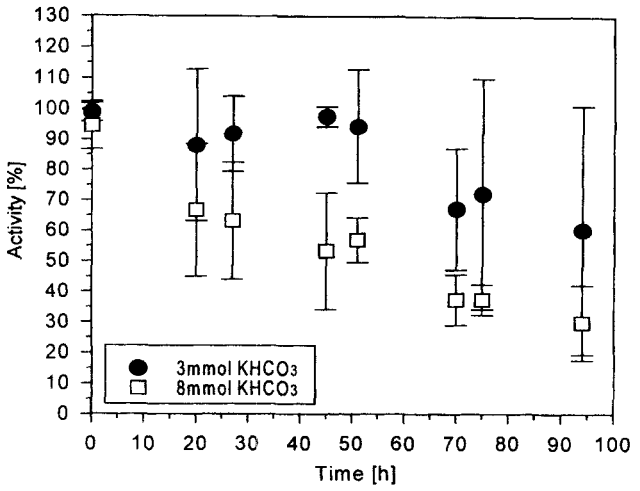

Figure 3. Influence of reaction time on remaining enzyme activity during a Thermoase PS160 catalyzed synthesis of Z-Aspartame at 40°C (2 mmol Z-Asp, 2 mmol Phe-OMe·HCl, 300 mg Thermoase P160, and 200 μL H_2O per reaction) with two different additions of $KHCO_3$.

Activity measured with hydrolytic enzyme assay. Measurements were performed during three different reactions. One hundred percent activity indicates the enzymatic activity at the start of the reaction. Activity measurements were done in at least duplicate, but the large scatter represents sampling problems from the mainly solid reaction mixture rather than assay error.

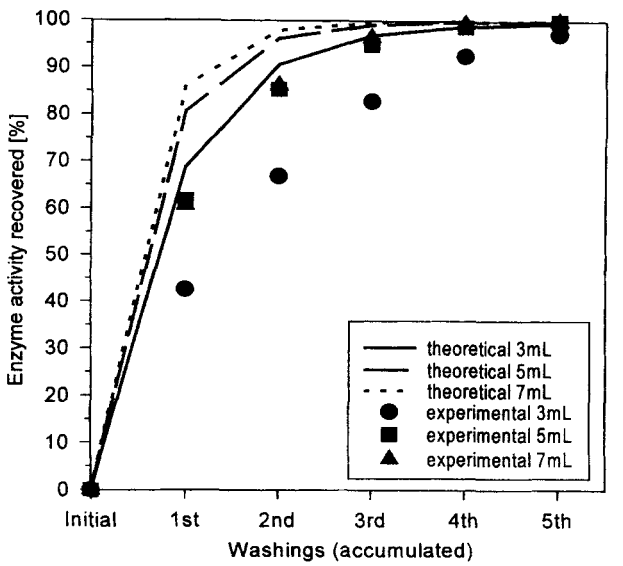

Figure 4. Experimental data vs. stoichiometric calculations for washing with three different amounts of water.

Experimental washing was carried out after a Thermoase PS160 catalyzed synthesis of Z-Aspartame (2 mmol Z-Asp, 2 mmol Phe-OMe·HCl, 3 mmol $KHCO_3$, 150 mg Thermoase P160, and 200 μL H_2O per reaction). By estimating the liquid volume recovered, the wet cake was found to contain around 1.2 mL H_2O. The stoichiometric calculations are based on this value, assuming the enzyme concentration to be equal in the filtrate and the cake water. Points are average of at least two washings.

per mol of each substrate (filled symbols). The activity decreased between 5 and 10% per day. We reached a yield of around 80% conversion after 24 h with an enzyme concentration of 150 g per mol substrates, so the reaction can be terminated without a large loss in enzyme activity. With the addition of 4 mol $KHCO_3$ (open symbols), it is clear that the enzyme is inactivated more rapidly. The remaining activity of around 65% after 24 h, however, suggests that irreversible enzyme deactivation is not the main reason for lower yield in this reaction. A change in the acid–base conditions as the reaction proceeds is probably the main factor (Erbelding et al., unpublished). The limited enzyme inactivation in the standard reaction, which uses a relatively high concentration of enzyme, means that enzyme recovery is likely to be economically viable. An alternative approach would be to use a lower enzyme concentration without recovery, accepting the longer reaction time and lower volumetric productivity.

Stages IV and V: enzyme recovery via product crystal washing

The washing of the catalyst from the product crystals consists of two stages: crystal washing and the separation of the product crystals from the enzyme solution. The first target is to add sufficient water to dissolve all the enzyme. The solubility is 115 mg Thermoase PS160 per 1 mL H_2O, so we used a washing volume of 1.5 L H_2O per mol reaction mixture, which should give 50 mg Thermoase per 1 mL H_2O, below saturation. Good mixing is also required at this stage to free all the enzyme trapped among the product crystals. For the subsequent solid–liquid separation, we chose a centrifuge as a model method because it guarantees fast separation and easy handling. This could also be carried out by any filtration device, preferably within the reactor. The results of the first two washings plotted in Figure 4 show rather low performance in comparison with the stoichiometric calculation, and we realized that enzyme must still be trapped in solid product crystals. The presence of this trapped enzyme was confirmed by an experiment with deliberately unsatisfactory mixing, so that large structures of product crystals remained during the first two washings. In this case, only 26.5% and 16.6% of enzyme were recovered for the first and second washings, respectively. However, the lower initial performance was overcome when the additional washings were applied, with recoveries of 21.6, 27.2, and 6.0%, as shown in Figure 4. It can also be seen from Figure 4 that a smaller amount of enzyme was recovered during the first two washings and recovery of Thermoase improved significantly after the second washing.

From Figure 4 it can be seen that the fifth washing did not improve the overall results significantly, and that there was only a small difference between the added volumes of 5 mL and 7 mL H_2O per cycle. Minimizing the volume and number of washings will reduce the loss of product by dissolution, so it seems sensible to use four washings with 5 mL. We implemented this for the washing of our standard reaction mixture at 2 mmol scale. The composition of the product streams before, during, and after washing is shown in Figure 5. We

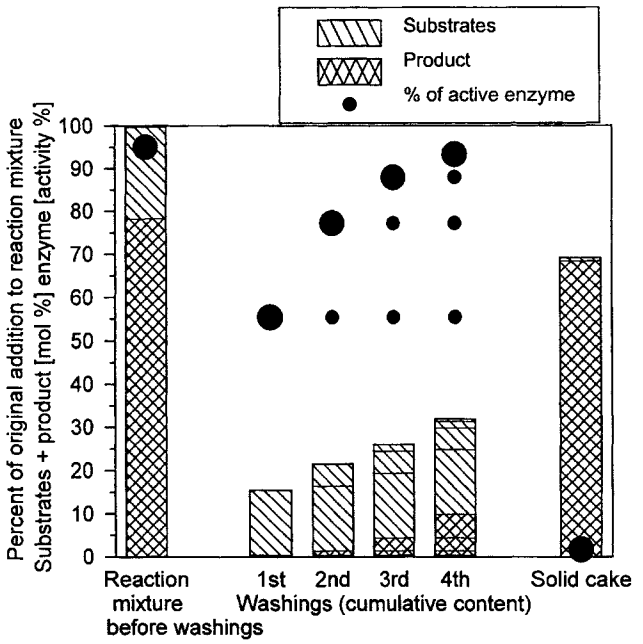

Figure 5. Comparison of product streams before, during and after washing.

Washing was carried out using 5 mL H_2O each cycle after a Thermoase PS160 catalyzed synthesis of Z-Aspartame (2 mmol Z-Asp, 2 mmol Phe-OMe·HCl, 3 mmol $KHCO_3$, 300 mg Thermoase P160, and 200 mL H_2O per reaction). Values are average of two experiments (substrates = remaining Z-Asp (Phe-OMe.HCl equal); Product = Z-Asp-Phe-OMe product (mol %)). Washings are completed (section in bars and smaller ● symbols represent contributions from each previous washing).

noted that the substrates are substantially more soluble in water than the product (at least 10-fold). Thus the washing procedure achieves a purification of the product without significant loss: the remaining cake contains less than 2% substrates with a loss of less than 15% of the originally formed product. In a scaled-up process the fate of the materials dissolved in the washings would also be important, including the inorganic salts. It would have to be considered whether it is economically sensible to recycle the substrate or recover the product.

Only about 1% of the enzyme activity is lost either in the remaining wet cake or through inactivation during the washing process. Since about 5% of the activity is lost by inactivation during the reaction, the overall recovery of enzyme was about 94%.

We also tested the performance of the mixer/reactor in the washing procedure after a 200-mmol reaction. At this scale we added 300 mL of water directly into the reactor. As a result, the mixer proved to be efficient in breaking the large aggregates of product, resulting in a fine suspension of product crystals. We recovered 56.0, 13.3, and 8.7% of enzyme activity in three 300 mL H_2O washing cycles. Note that the total enzyme recovery (76.0%) is less than in Figure 5 (87.0%), because we had to use less water per unit product.

Stage VI: enzyme reconcentration by ultrafiltration

In scale-up applications, ultrafiltration is probably the most efficient method for concentrating the enzyme solution from the washing process. The final concentrate, however, is required to be an enzyme suspension, above the solubility limit. This might be expected to cause some problems for an ultrafiltration process. Therefore we investigated the performance of ultrafiltration with varying enzyme concentrations, particularly above the protein saturation of around 23 mg·mL^{-1}, and up to 300 mg·mL^{-1}. The first row in Table 3 and the circles in Figure 6 are for ultrafiltration of the crude Thermoase PS160. Thermoase PS160 contains around 20% (w/w) protein (Bio-rad analysis), whereas thermolysin contains around 60% (w/w). About 50% of the weight in Thermoase could be recovered in the ultrafiltration filtrate, so the remaining 30% of the nonprotein content of Thermoase must be polymers of a high molecular weight, which cannot cross the membrane.

Table 3. Results of Ultrafiltration Processes with Starting, Final and Extrapolated Protein Concentrations

Protein Loading, mg	Initial Vol., mL	Final Conc. mg·mL^{-1}	Time h	Extrapolated Process Time,* h
600**	300	48	6.75	12
1,230†	300	100	6.25	8
1,230††	150	123	5	6
3,360‡	300	258	19	19.5

*Time is extrapolated to give estimated process time for the ultrafiltration process to reach a protein concentration of 300 mg mL^{-1}, as required by solid-to-solid reaction.
**Protein straight from the bottle (protein content of Thermoase PS160 equals 20% (w/w)).
†Protein recovered from solid-to-solid reaction (20 mmol scale).
††Protein recovered from solid-to-solid reaction, concentrated by ultrafiltration and rediluted to initial concentration.
‡Protein recovered from solid-to-solid reaction (200 mmol scale).

We also found that there was a significant difference in ultrafiltration performance between Thermoase straight from the bottle and that recovered by crystal washing after reactions (rows one and two in Table 3, solid and empty symbols in Figure 6). Even though the latter started at around double the protein concentration (4.1 mg mL^{-1} instead of 2 mg

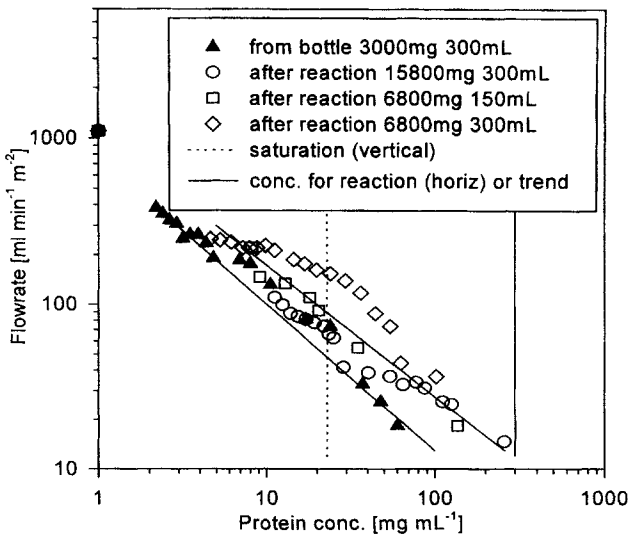

Figure 6. Flow rate of ultrafiltration processes vs. protein concentration with different enzyme loading.

mL^{-1}), it took approximately the same time to concentrate the starting volume from 300 mL to 12 mL. The concentrates of the recovered enzyme were also observed to be noticeably less viscous at equal concentrations. This is probably because some of the nonprotein polymers present in crude Thermoase are left in the washed product crystals. To test the influence of small molecules present in the washings, the concentrated enzyme batch (row two in Table 3) was rediluted and processed again. The ultrafiltration performance was essentially unchanged (row three), which showed that small molecules had little effect.

Table 3 also shows the estimated process times it takes to reach the required protein concentration of 300 mg mL^{-1}. Because of the minimum final volume obtainable in our device, we could not reach this target from the lower starting concentrations, so extrapolation of the lines in Figure 6 was required. To test the final stages of the concentration process, we used enzyme at an initial protein concentration of 11 $mg \cdot mL^{-1}$, obtained from the first washing of a reaction mixture at 200 mmol scale. Due to the relatively small membrane surface, this ultrafiltration process took 19 h (fourth row in Table 3 and empty circles in Figure 6) to achieve the final enzyme concentration. Over this period there was significant loss of enzyme activity. In practice, a larger ratio of membrane surface to volume would be desirable to reduce the required process time. It should be pointed out that we cannot exclude the possibility that enzyme precipitation leads to some of the observed inactivation.

As Figure 6 shows, ultrafiltration of the recovered enzyme should reach the target concentration with a final flow rate of 10 $mL \cdot min^{-1} \cdot m^{-2}$. The track of the points for enzyme straight from the bottle shows a significantly lower flow rate at any given protein concentration. It is interesting to note that there is no obvious discontinuity at the point where the protein reaches saturation. The protein particles that were observed to precipitate above this concentration did not seem to reduce flux any more than the same concentration of dissolved protein.

Conclusion

We have reported some key features for scale-up of enzymatic solid-to-solid peptide synthesis where enzyme recovery and recycling are required. A simple anchor stirrer with baffles proved to be sufficient for the various stages of mixing required prior to and after the reaction. The reaction mixture varied from solid to viscous paste, and finally to a liquid with suspended product crystals. Enzyme can be washed from the product crystals, and the washings concentrated back to the required enzyme suspension by ultrafiltration. Further study of ultrafiltration (such as increased membrane area and low temperature operation) is needed to reduce the loss of enzymatic activity.

A future process design might consist of a small mixer for the following stages: initial mixing; the neutralization reaction; the drying of the reactants; and the blending of the aqueous enzyme suspension with the reactants. A mixer with a jacketed body and the possibility of sparging dry air can fulfill all of these tasks. The reaction itself could occur in a jacketed extruder with a residence time of around 24 h. The product then would be discharged into the enzyme washing unit, which is in line with the ultrafiltration unit. Depending on the required scale, the whole process could even be run in a continuous mode.

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